New approaches in the neoadjuvant systemic therapy of breast cancer

Ph.D. Thesis

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List of full papers that served as the basis of the Ph.D. thesis

I. **Rusz O**, Vörös A, Varga Z, Kelemen G, Uhercsák G, Nikolényi A, Ormándi K, Simonka Z, Kahán Z
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LAPTM4B gene copy number gain is associated with inferior response to anthracycline-based chemotherapy in hormone receptor negative breast carcinomas
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III. Tőkés AM+, **Rusz O**+, Cserni G, Tóth E, Tőkés T, Vízkeleti L, Kószó R, Kahán Z, Kulka J, Szállási Z
Influence of mutagenic *versus* non-mutagenic pre-operative chemotherapy on the immune infiltration of breast cancer
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Other articles

I. **Rusz O** and Kahán Z
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Pathology & Oncology Research 19:1-10 (2013)
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II. Kővári B, **Rusz O**, Schally AV, Kahán Z, Cserni G
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Emlőrák neoadjuváns hormonterápiájával szerzett tapasztalataink
Orvostovábbképző szemle – különszám 2012. november – Magyar Klinikai Onkológiai Társaság VIII. kongresszusa

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Bartyik K, Bessenyei Zs, Bihari Z, Csada E, Dank M, Együd Zs, Fábián G, Hideghéty K, Kahán Zs, Kószó R, Krenács L, Maráz A, Nagy N, Oláh J, Pávics L, Peták I, Rusz O, Sántha D, Szabó Cs, Szabó Z, Széll M, Tordai L, Varga L, Végváry Z
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List of abbreviations

ABD	axillary block dissection
AC	doxorubicin plus cyclophosphamide
AC+T	doxorubicin plus cyclophosphamide and sequential docetaxel
AI	aromatase inhibitor
ASCO	American Society of Clinical Oncology
BRCA	breast cancer gene
CAP	College of American Pathologists
CBP	carboplatin
CDDP	cisplatin
CEN8	chromosome 8 centromeric region
CI	95% confidence interval
CMF	cyclophosphamide plus methotrexate plus 5-fluorouracil
CR	complete response
DCIS	ductal carcinoma in situ
DMFS	distant metastasis-free survival
EC	epirubicin plus cyclophosphamide
EC+T	epirubicin plus cyclophosphamide and sequential docetaxel
ER	estrogen receptor
ESMO	European Society for Medical Oncology
ET	endocrine therapy
FAC	5-fluorouracil plus doxorubicin plus cyclophosphamide
FAC+T	5-fluorouracil plus doxorubicin plus cyclophosphamide and sequential docetaxel
FEC	5-fluorouracil plus epirubicin plus cyclophosphamide
FEC+T	5-fluorouracil plus epirubicin plus cyclophosphamide and sequential docetaxel
FFPE	formalin fixed paraffin embedded
FISH	fluorescent in situ hybridization
GnRH	gonadotropin releasing hormone
HER2	human epidermal growth factor receptor 2
HMGB1	high-mobility-group box 1
HR	hormone receptor
HRD	homologous recombination deficiency
IHC	immunohistochemistry

IQR	interquartile range
ISH	in situ hybridization
LAPTM4B	lysosomal-associated transmembrane protein 4b
MHC	major histocompatibility complex
NCCN	National Comprehensive Cancer Network
NET	neoadjuvant endocrine therapy
NK	natural killer
NST	neoadjuvant systemic therapy
OR	odds ratio
pCR	pathological complete response
PD-1	programmed death 1
PDL-1	programmed death-ligand 1
PFS	progression-free survival
PgR	progesterone receptor
pNR	pathological non-response
post-StrTIL	stromal tumor infiltrating lymphocyte in surgical sample after pre-operative therapy
pPR	pathological partial response
PR	partial response
pre-StrTIL	stromal tumor infiltrating lymphocyte in core biopsy before pre-operative therapy
ROC	receiver operating characteristic
RT-PCR	real-time polymerase chain reaction
SNB	sentinel lymph node biopsy
StrTIL	stromal tumor infiltrating lymphocyte
TAC	docetaxel plus doxorubicin plus cyclophosphamide
TAX	paclitaxel
TEX	docetaxel plus epirubicin plus capecitabine
TIL	tumor infiltrating lymphocyte
TLR	toll-like receptor
TNBC	triple-negative breast cancer
TOP2A	topoisomerase-II-α
WHO	World Health Organization

1. Introduction

Treatment of breast cancer is one of the most complex areas in oncology. Therapeutic approaches of breast cancer include the treatment of local disease with surgery and radiotherapy or both, and systemic therapy with chemotherapy, endocrine therapy (ET), targeted therapy or the combinations of these. The treatment strategy is influenced by several factors, first of all the stage and pathologic characteristics of the disease, patient comorbid conditions, age, menopausal status and, last but not least, the cosmetic outcome in accordance with the patient's expectations.

For those women who desire breast conserving surgery, but it is not possible due to the size of the tumor or who are not suitable for surgery due to inoperable tumor status (such as inflammatory breast cancer or other T4 tumors), neoadjuvant systemic therapy (NST) is considered. Efficient NST may decrease the radicality of not only of surgery, but also that of radiotherapy. Another benefit of NST is the prognostic information provided on the basis of therapy response. A good response predicts favorable outcome, and the prognosis may therefore be estimated more accurately and earlier than in the adjuvant setting. Hence nowadays NST should be considered in all cases in which adjuvant systemic therapy is necessary, or if by giving that as primary intervention, any benefit may be expected [1–3]. The complete disappearance of any invasive cancer from the breast and lymph nodes, referred to as a pathological complete response (pCR), means excellent prognosis [4–6]. The Food and Drug Administration has proposed the use of the rate of pCR as a surrogate marker, and NST a strategy that could be utilized for the accelerated approval of new drugs [7].

In neoadjuvant setting, the same systemic therapeutic regimens can be applied as in adjuvant setting and the therapeutic decision is based on the immuno- and molecular-pathologic determination of the tumor tissue in core biopsy. Currently, examinations of the following biomarkers are essential in all breast cancer cases: the expression of the estrogen receptor (ER), progesterone receptor (PgR) by immunohistochemistry (IHC), as well as, the human epidermal growth factor receptor 2 (HER2) expression by IHC and/or the copy number of the gene assessed by the in situ hybridization (ISH) technique.

Breast cancer cases that have at least 1% of cells staining positive for ER and/or PgR, may be considered hormone receptor (HR)-positive. Breast cancers are classified as HER2-positive if scored as 3+ by IHC or if the amplification of the HER2 gene is demonstrated by an ISH method [2, 8, 9]; triple-negative tumors lack the expression of HR and HER2.

This classification serves as a guide for oncological therapy decision. In case of triple-negative breast cancer (TNBC) or HER2-positive cases, combined chemotherapy is the standard therapy alone or in the latter case in combination with anti-HER2 agents (trastuzumab or trastuzumab plus pertuzumab). In HR-positive tumors, while the use of ET is strongly recommended, the necessity of chemotherapy is not obvious in all cases.

ER-positive and HER2-negative breast cancer is the most common subtype, approximately 73% of all breast carcinomas show these features. This phenotype is more frequent with age, and shows more favorable clinical prognosis (as compared to that of HER2-positive and triple-negative cases), endocrine responsiveness and less sensitivity to chemotherapy [5, 10, 11]. ET is strongly suggested independently of the therapeutic setting (adjuvant, neoadjuvant or palliative). As curative therapy, tamoxifen or the aromatase inhibitors (AIs) are accepted in combination with or without gonadotropin-releasing hormone (GnRH) agonist depending on the sex hormonal status.

Tamoxifen was the first registered ET (1978) for the treatment of breast cancer. It is a selective estrogen receptor modulator, binds to the ERα as an antagonist and to ERβ as an agonist. ERα is responsible for many of the effects of estrogen on normal and cancerous breast tissue, through ligand-activated transcriptional regulation and by acting as a component of membrane and cytoplasmic signaling cascades [12]. Adjuvant therapy with tamoxifen almost halves the rate of disease recurrence and reduces the annual breast cancer death rate by one-third, making a significant contribution to the 25-30% decrease in breast cancer mortality achieved in the past two decades [13].

Subsequently, other new ETs have been developed that target estrogen biosynthesis, the aromatase inhibitors. Currently, three members of the new generation AIs are available in medical uses: the steroidal AI exemestane, is an irreversible inhibitor of the aromatase enzyme, whereas the non-steroidal AIs, letrozole and anastrozole are competitive antagonists of that.

In premenopausal women, the ovaries are the primary site of estrogen production. AIs are not capable of blocking ovarian estrogen synthesis completely; therefore they cannot be used as monotherapy unless in combination with ovarian suppression therapy, such as goserelin or leuprorelin (GnRH agonists) providing the full blockade of estrogen synthesis [14].

Unlike chemotherapy, ET is not able to enhance the apoptosis of cancer cells, instead, hinders or decelerates cell proliferation. Consequently, for maximum therapeutic effect, their prolonged administration is necessary, it means five or ten years in the adjuvant setting.

From the first clinical use of ETs, almost 10 years have passed until their neoadjuvant application. At the beginning only elderly women received NET [15–17] with the aim of avoiding chemotherapy and its side-effect, which is poorly tolerated by this population. Nowadays, it is generally accepted that not all HR-positive cases need chemotherapy and the therapy decision should depend on tumor characteristics rather than on age. Likewise, in the adjuvant setting in clinically low risk cases, such as pT1a/b, grade 1, ER high, N0 and similar ones, chemotherapy is not indicated under any circumstances. However, for the decision about adjuvant therapy in patients with higher clinical risk disease, the use of a multigene test, such as the 21-gene RT-PCR assay (OncotypeDX®) is recommended by all international guidelines (National Comprehensive Cancer Network - NCCN, American Society of Clinical Oncology - ASCO, St. Gallen).

OncotypeDX® provides prognosis and predicts benefit of a certain systemic therapy. In clinical practice, three risk groups are distinguished by the assay: in cancers with low recurrence score (<18), the risk of relapse is very low, and the use of exclusive endocrine therapy is sufficient; cancers with intermediate recurrence score (18-30) sole endocrine therapy or sequential chemotherapy-endocrine therapy are individually selected; cases with high risk recurrence score (>31) show poorer prognosis, and the addition of chemotherapy significantly improves outcome. In fact, the recent results of the TAILOR-x study indicate that below the risk score of 26, there is no benefit of adjuvant chemotherapy among small and node-negative HR-positive and HER2-negative cases [18].

In node-positive cases, the use of genetic tests in the decision about chemotherapy has a less clear role [5]. The Panel of 15th St. Gallen International Expert Consensus Conference (2017) considered the node positive status as a strong negative prognostic factor regardless of the gene expression signature, thus, did not universally endorse the use of gene expression signatures for making treatment decision on adjuvant chemotherapy in node-positive cases. The Panel agreed that, gene expression assays (such as the 21-gene recurrence score) are useful tools, but the classification based on routine histopathology is still clinically valuable, and should be the basis of assisting treatment decision [9]. They subcategorized HR-positive and HER2-negative breast cancers according to the expression of Ki67 as evaluated by IHC or the grade: Luminal A-like tumors are typically of low grade, strongly ER/PgR-positive, HER2-negative and have a low proliferative fraction while Luminal B-like tumors are ER-positive but may have variable degrees of ER/PgR expression, are of higher grade, and have a higher proliferative fraction. The last ESMO guideline (2015) uses the 13th St. Gallen Conference classification: Luminal A (ER-positive/HER2-negative, PgR≥20%, Ki67<20%),

Luminal B1 (ER-positive/HER2-negative, PgR<20% or Ki67≥20%), Luminal B2 (ER-positive/HER2-positive), HER2-positive (ER/PgR-negative and HER2-positive) and Basal-like (triple-negative) [19]. ASCO recommends the use of the proliferation marker Ki67, to guide the choice of adjuvant chemotherapy with caution.

There are limited experiences with genetic tests in neoadjuvant therapy and their use is not yet evidence-based for decision making. Hence the decision on NET is usually based on conventional pathological and clinical data, and should be offered in those cases, in which the best response is anticipated, mostly the Luminal A-like tumors.

Expanding clinical experiences indicate a pCR ratio of 0-17.5% in association with the length of NET [20, 21]. This ratio is surprisingly low as compared to the pCR rate of around 40% in HR-negative cases after neoadjuvant chemotherapy [5]. Nonetheless, the long-term outcome is excellent in spite of residual cancer in this group of patient [22]. These experiences raise the question, whether pCR may be considered as a reliable end-point of treatment response in this therapeutic modality at all.

In contrast, HR-negative breast cancers show less favorable outcome, and the only available therapy is chemotherapy (alone or in combination with anti-HER2 therapies based on the HER2 status). In Hungary, in accordance with the international guidelines, the most favorable neoadjuvant therapy regimens are anthracycline-containing (doxorubicin, epirubicin), platinum-containing (cisplatin, carboplatin) and taxane-containing (paclitaxel, docetaxel) combinations. However, there is no existing reliable biomarker to guide the decision about the chemotherapy regimen. The breast cancer gene-1 and -2 (*BRCA1/2)* genes are members of the homologous recombination DNA repair pathway, which is responsible for the error-free repair of DNA double strand breaks. The hereditary mutations of these genes increase the risk of cancers of the breast, ovary, pancreas or prostate. The tumors with *BRCA1* or *BRCA2* mutation are considered having high sensitivity to DNA crosslinking agents such as platinum [23]. Although von Minckwitz et al. observed a higher ratio of pCR in the *BRCA* mutant cases than in the wild-type cases, they experienced a similar pCR ratio in the carboplatin arm as in the non-carboplatin arm [24]. The dysfunction of the homologous recombination DNA repair pathway occurs in sporadic tumors as well, which are not carrying *BRCA1*/2 mutations. The Homologous Recombination Deficiency (HRD) score is an unweighted sum of three independent DNA-based measures of genomic instability and reflects the function of the homologous recombination repair pathway. The HRD score seemed to be a predictor of platinum sensitivity [25], but its specificity was disproved by Sharma et al. [26]. According to their experience, adjuvant doxorubicin and

cyclophosphamide treatment produced equally good disease-free survival in the HRD score-positive cases [26].

The mechanism of anthracycline resistance has been an important question from its first clinical use (1974). The cellular distribution of the anthracyclines has been extensively studied, first by utilizing their fluorescent properties. Most likely, these agents can enter the cells through simple diffusion [27], then they bind to the proteasomes and by an ATP-dependent nuclear pore-mediated mechanism get transported into the nuclei [28]. Both doxorubicin and epirubicin are weak bases consequently they can accumulate in acidic intracellular compartments, such as the lysosomes. Several studies have reported that resistant cancer cells are able to accumulate significantly more anthracyclines in the cytoplasmic organelles, resulting in reduced nuclear drug accumulation and decreased cytotoxicity [29]. Nonetheless, the potential molecular regulation of the drug sequestration in acidic lysosomes was unclear for a long time.

In a recently published article, the amplification of 8q22 and the accompanying overexpression of the lysosomal-associated transmembrane protein 4b (LAPTM4B) was associated with worse prognosis and worse therapy response to anthracycline-based chemotherapy in patients with ER-negative breast cancer [30].

LAPTM4B is a lysosomal membrane protein that comprises four transmembrane domains. The lysosome membrane-stabilizing properties of LAPTM4B is responsible for retaining the drug in the lysosome and decrease its nuclear translocation by sequestering it in the cytoplasmic compartment. Knockdown of LAPTM4B not completely but significantly weakened the capability of lysosomes to retain doxorubicin. The preservation of lysosome membrane integrity by LAPTM4B also prevents cathepsin release and the following caspase-mediated apoptosis which was detectable after doxorubicin, but not after exposure to taxol [31]. Based on these results, LAPTM4B seems a potential biomarker to predict anthracycline sensitivity.

In HR-negative cases, the complete disappearance of the tumor after neoadjuvant chemotherapy is an important surrogate end-point of favorable prognosis. Nevertheless, some cases with residual disease achieve long-term survival. Preclinical studies have suggested that cytotoxic agents may partly exert their antitumor activity by inducing immune response against tumor cells [32–35]. This triggered immune response can keep prolonged control over the residual tumor cells. Each cytotoxic drug interacts with the immune system in its own complex manner. Not all, but certain chemotherapeutic agents, for instance doxorubicin and oxaliplatin, induce immunogenic cell death. This type of apoptosis is characterized by the

translocation of calreticulin from inside the endoplasmic reticulum to the cell surface, where it provides “eat me” signals for specialized antigen-presenting cells, and in particular for dendritic cells [34]. Dendritic cells capture the apoptotic tumor cells and cross-present their major histocompatibility complex (MHC) class I molecules as antigens derived from the internalized dying cells for recognition by CD8 T-cells [36], thus eliciting the anticancer immune response [35]. In addition, as a consequence of chemotherapy (e.g. doxorubicin), dying tumor cells release high-mobility-group box 1 (HMGB1) protein. The interaction of HMGB1 with the toll-like receptor 4 (TLR4) expressed by the dendritic cells, is necessary for the efficient cross-presentation of antigens derived from apoptotic cancer cells [32].

The other way doxorubicin can inhibit tumor-induced immune tolerance is the downregulation of the programmed death-ligand 1 (PD-L1) in tumor cells [37]. PD-L1 is one of the two ligands of the programmed death 1 (PD-1) receptor, a co-inhibitory molecule on T-cells. Through the display of PD-L1, tumor cells can negatively regulate T-cell activation, and escape immune surveillance [38]. Basically, cyclophosphamide was known for its immunosuppressive nature, but the preclinical findings proved this property as dose- and schedule-dependent. Low-dose cyclophosphamide contributes to antitumor immunity by suppressing regulatory T-cell function (regulatory T-cells mediate immunosuppressive networks), restoring the proliferative capacity of effector T-cell and natural killer (NK) cell cytotoxicity. In contrast, high-dose cyclophosphamide shows immunosuppressive effects and works solely through cytotoxicity [38, 39]. Paclitaxel, doxorubicin and cisplatin sensitize tumor cells to cytotoxic T-cells by making tumor cells permeable to granzyme B (a protease by which cytotoxic T-cells may destroy target cells) [40].

In addition to these direct immunomodulatory properties, the mutagenic effects of chemotherapies on tumor cells may also participate in antitumor immune activation.

It is likely that, only one or a few mutations per tumor are immunodominant, and the tumors with a higher mutational burden have an increased likelihood of bearing a highly immunogenic mutation [41]. Breast carcinomas carrying about one mutation/Megabase on average, have a modest mutational burden, at least when compared to melanomas or lung adenocarcinomas in which as many as 100 mutations/Megabase may be possible [42]. Nevertheless, the mutation rate may be raised by chemotherapy itself thereby enhancing immunogenicity. These acquired mutations are often translated into altered proteins including novel peptide sequences, which may become neo-epitopes. These neo-epitopes are believed to be particularly immunogenic because they are not encoded in the normal genome of the

individual patient, thus reactive T-cells are not subjected to central tolerance. Recognition of neo-epitopes by cytotoxic T-cells may lead to immune-mediated tumor regression.

Szikriszt et al. [43] previously experimentally classified current chemotherapy regimens as highly mutagenic (cisplatin), moderately mutagenic (cyclophosphamide) and marginally/non-mutagenic (paclitaxel, doxorubicin and gemcitabine). They found that cisplatin induces 20-fold, while cyclophosphamide therapy induces 5-fold more mutations than taxanes or anthracyclines. Perhaps, the different mutagenic properties may also contribute to different immunomodulatory effect of chemotherapies [43].

Since neoadjuvant systemic therapy is a rational option in breast cancer serving tailored therapy, it is a more and more accepted and practiced treatment modality. This setting provides at the same time a unique opportunity to gain special observations for further improve the outcome.

2. Aims

2.1 To evaluate the benefit of one-year neoadjuvant endocrine therapy in HR-positive and HER2-negative breast carcinomas, as well as, to investigate the association between the characteristics of the residual tumor and disease outcome in this patient population.

2.2 To examine the *LAPTM4B* copy number by fluorescent in situ hybridization technique in breast carcinoma samples obtained before treatment and its role in therapy response to anthracycline-based therapy *versus* non-anthracycline-based treatment. We hypothesized, that the extra copy of *LAPTM4B* has a negative predictive value to anthracycline-containing therapy.

2.3 To compare the effects of preoperative therapy with the taxane-anthracycline combination (as low mutagenic regimen) *versus* cyclophosphamide-based chemotherapy (as moderately mutagenic regimen) *versus* taxane-platinum chemotherapy (as high mutagenic regimen) on the percentage of stromal tumor infiltrating lymphocytes. We hypothesized that induction of a higher number of mutations and neo-epitopes with mutagenic chemotherapy might result in stronger immune reactions in the tumor microenvironment, and this could be reflected by the increase of lymphocytic infiltration.

3. Patients and methods

3.1 Study populations

One-year neoadjuvant endocrine therapy in breast cancer

Forty-two patients having received neoadjuvant endocrine therapy (NET) between 04/2005 and 01/2014 were included. Patients were eligible for NET if they had histologically confirmed ER-positive and PgR-positive, invasive breast cancer in stages II or III (UICC/AJCC TNM classification system *vs*. 7.0) and if imaging examinations, including chest X-ray, abdominal ultrasonography and bone scan, ruled out distant metastases.

The initial local/regional tumor status and that after NET were evaluated through physical examination, mammography, ultrasonography and breast MRI in some cases. The initial tumor size was determined from the size of the mammographic abnormality, or, if there was no abnormality, via the MRI image. Prior to the start of NET, three tissue cylinders for histopathological examinations were taken from each patient with a 16 G core needle. In three of four patients with bilateral breast cancer, only fine needle aspiration proved the existence of cancer in the less advanced tumor. In potential candidates for breast conserving surgery, a clip (O-Twist-Marker, BIP Biomed. Instrumente & Produkte GmbH, Germany) was inserted into the tumor for visualization purposes before the NET.

Treatment with letrozole (n=33, postmenopausal group), or with goserelin plus letrozole (n=7) or with goserelin plus tamoxifen (n=2) (premenopausal group) was planned for 1 year. The therapeutic response was monitored by palpation every 3 months, or with imaging if necessary. In the event of progression, NET was replaced by neoadjuvant chemotherapy; if the therapeutic effect was judged to be insufficient, letrozole was replaced by tamoxifen. After 1 year of NET, surgery was designed individually with regard to the post-therapy imaging results and the initial tumor stage. The imaging response based on mammography was evaluated in accordance with the World Health Organization (WHO) criteria [44]. Sole sentinel lymph node biopsy at the time of surgery was aimed at in cases of clinical lymph node negativity. If the sentinel lymph was found to be metastatic, complementary axillary block dissection was performed.

On the basis of the volume of the remaining tumor in the surgical specimen, the following risk groups were constructed:

Group 1: no invasive tumor (stage 0)

Group 2: small-volume residual tumor (stages IA-IIA)

Group 3: large-volume residual tumor (stages IIB≤) + cases with clinical progression.

Following surgery, post-operative radiotherapy and adjuvant systemic therapy were applied in accordance with the institutional guidelines.

LAPTM4B gene copy number gain and response to anthracycline-based chemotherapy in hormone receptor negative breast carcinomas

A total of 143 cases with HR-negative breast carcinoma were enrolled and were analyzed in two different cohorts. The study was ethically approved by the Semmelweis University Institutional Review Board (SE-TUKEB 120/2013).

The first cohort included 69 core biopsies of HR-negative (64 TNBC and 5 HER2-positive) primary breast carcinoma cases diagnosed between 2004 and 2016, who received at least two cycles of chemotherapy, and then underwent surgery. Patients were eligible for neoadjuvant therapy, if they had histologically confirmed invasive breast cancer and imaging examinations ruled out distant metastases.

Fifty out of 69 patients (72.5%) were treated with anthracycline-based neoadjuvant chemotherapy (mainly in combination with taxane), whereas 19 patients (27.5%) represented the control arm receiving non-anthracycline-based (mostly platinum in combination with taxane) chemotherapy.

The second cohort included 74 samples of surgically removed HR-negative breast carcinomas (39 TNBC, 27 HER2-positive, and 8 with unknown HER2 data). Patients in this cohort were treated with chemotherapy in the adjuvant setting between 1999 and 2006. Out of these patients, 57/74 (77.0%) received anthracycline-based therapy (22.8% in combination with a taxane) and 13/74 (17.6%) received non-anthracycline-based therapies (as control arm), mainly cyclophosphamide plus methotrexate plus 5-fluorouracyl (CMF) regimen. In 4/74 (5.4%) cases, no treatment data were available.

Influence of mutagenic versus non-mutagenic pre-operative chemotherapy on the immune infiltration of breast cancer

Samples of 112 patients diagnosed with breast carcinoma and treated with pre-operative chemotherapy in four Hungarian institutions (National Institute of Oncology, Onco-Radiology Center of Bács-Kiskun County Teaching Hospital, Semmelweis University and University of Szeged) between 2005 and 2017 were selected and studied retrospectively. The inclusion criteria were as follows: availability of both a core biopsy and surgical tumor sample, known clinical and treatment data, at least 2 cycles of chemotherapy administered before surgery, residual tumor after pre-operative chemotherapy. All patients underwent

breast surgery. Of the 112 cases, 103 received chemotherapy plus surgery with curative intent, while 9 cases had bone metastases at the beginning of pre-operative chemotherapy.

Based on the HR and HER2 statuses, cases were classified into four different subtypes.

According to the type of pre-operative chemotherapy, the patients were grouped into platinum-based (n=28), cyclophosphamide-based (n=42) and anthracycline-based (n=42) groups. In platinum-based group, 21 patients were treated with carboplatin plus docetaxel or paclitaxel and 7 patients received cisplatin plus docetaxel or paclitaxel. In cyclophosphamide-based group, 57.1% (n=24) received anthracycline (epirubicin (E) or doxorubicin) plus cyclophosphamide (C) in combination with or without 5-fluorouracil (F) followed by docetaxel. The other commonly used treatment regimen in this group was FEC without following taxane therapy in 35.7% (n=15) of the cases. In anthracycline-based group, all cases were treated with anthracycline plus taxane combination (of those 32/42 received epirubicin plus docetaxel or paclitaxel). All treatment regimens were of conventional doses and schedules, and selected based on valid international guidelines.

3.2 Pathology

The tumor histological type was defined according to the most recent WHO classification [45].

IHC data were collected from the original pathology reports. Each pathology centrum used its own IHC protocol in accordance with the national guidelines. HR status was scored according to the current Hungarian Guideline [46] and the ASCO/CAP's recommendations [47]. A case was considered HR-negative if the expression of ER and PgR was less than 1%. HER2 positivity was evaluated conforming to the United Kingdom recommendations [48].

Ki67 staining was interpreted per the recommendations of the international working group [49]. For the determination of the topoisomerase-II-α (TOP2A) status, nuclei of 50 tumor cells were counted under the microscope and the proportion of stained cells was recorded. In both cases, a cut-off value of 15% was applied to separate negative (≤15%) and positive samples (>15%) in cases who received NET.

The degree of response to neoadjuvant therapies was categorized following Pinder et al. [50]. A complete pathological response (pCR) comprised either (i) no residual carcinoma in the breast and lymph nodes or (ii) no residual invasive tumor but ductal carcinoma in situ (DCIS) present in the breast and absence of any residual invasive tumor in the lymph nodes. A partial response to therapy (pPR) meant either (i) minimal residual disease/near total effect (e.g. <10% of tumor remaining) or (ii) evidence of response to therapy but with 10-50% of

tumor remaining or (iii) >50% of tumor cellularity remaining evident, when compared with the previous core biopsy sample, although some features of response to therapy being present. A case was consider non-responder if there was no evidence of response to therapy (pNR).

FISH analysis of LAPTM4B copy number

Interphase FISH analysis was used to evaluate the copy number status of *LAPTM4B* gene. The 5 μm thick formalin fixed paraffin embedded tissue (FFPE) sections were mounted onto Superfrost Plus positively charged slides, deparaffinized and rehydrated in distilled water. For antigen retrieval, sections were incubated in citric acid based antigen unmasking solution (Vector Laboratories, Inc. Burlingame, CA, USA) at 95 °C for 20 min. Cell lysis was established by incubating the sections in Triton X-100 (AppliChem GmbH, Ottoweg 4, 64291 Darmstadt, Germany) –SSC solution at 65 °C for 30 min. Sections were then subjected to digestion in pepsin solution, for 12 min at 37 °C, then washed twice in SSC for 5 min. ZytoLight® FISH-Tissue Implementation Kit (ZytoVision GmbH, Bremerhaven, Germany) was used in prehybridizational steps. Sections were air dried prior to denaturation at 73 °C for 10 min. Hybridization was performed using 4 μl of custom-made, Texas Red/FITC dual labelled *LAPTM4B*/CEN8q FISH probes (Abnova Corp., Taoyuan City, Taiwan) per slide at 37 °C for 16-18 h in an automated hybridization chamber (ZYTOMED Systems GmbH Berlin, Germany). Slides were then immersed in wash buffer SSC for 30 min at 45 °C, rinsed in water for 10 min, air-dried. Cell nuclei were counterstained with DAPI in antifade solution (Vector Laboratories, Inc. Burlingame, CA, 94010, USA).

The Leica DM RXA fluorescent microscope equipped with Leica DFC 365FX high performance CCD camera (Leica Microsystems GmbH, Wetzlar, Germany) and with DAPI long-pass, FITC and Texas Red filters was used to evaluate the hybridization results.

Areas with well-separated cell nuclei and overall good hybridization signals were selected for analysis. Minimum two FISH images per case were digitally captured at 63x magnification.

For each case, red (*LAPTM4B*) and green (CEN8 centromeric region) fluorescent signals were counted separately in at least 50 non-overlapping interphase nuclei. Based on these data the following parameters were calculated: average *LAPTM4B* copy number/cell, average CEN8q copy number/cell, *LAPTM4B*/CEN8q ratio, average *LAPTM4B* copy number/cell in amplified cell population and percentage of polysomic or amplified cells.

TIL analysis

FFPE blocks of core biopsies and surgical specimens were retrieved from the four pathology departments (Surgical and Molecular Tumor Pathology Center, National Institute of Oncology and the Departments of Pathology, Bács-Kiskun County Teaching Hospital, Semmelweis University and University of Szeged).

4 μm sections of representative tumor blocks were stained with hematoxylin and eosin. The percentage of stromal tumor infiltrating lymphocytes (StrTIL) was evaluated according to the recommendation of International TILs Working Group 2014 [51]. Histopathologic evaluation of StrTILs was performed by GCs, AMT, AV, ET and JK. Controversial cases were reevaluated and discussed.

3.3 Statistical analysis

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The normality of the data was controlled by the Shapiro-Wilk test.

In the first study with NET, the associations between age, initial ER, PgR, Ki67 and TOP2A expression as continuous variables and the three risk groups were analyzed by the one-way ANOVA test, whereas the menopausal status, grade, Ki67 and TOP2A as categorical variables were investigated by Fischer's exact test. In order to examine the changes in the tumor features after NET, the paired sample t-test and the McNemar test were used for the continuous and categorical variables, respectively. Binary univariate logistic regression models were applied to examine the potential predictive role of the expression of predictive markers.

Non-parametric tests: Fisher's exact, Kruskal-Wallis, Mann-Whitney-Wilcoxon tests were used to compare the *LAPTM4B* and CEN8q copy number to the clinicopathological data of the primary tumors, including therapy response in the neoadjuvant cohort and distant metastasis formation in the adjuvant cohort as end-points.

The association between changes in StrTIL and clinicopathological variables (type of pre-operative chemotherapy received, grade, immunohistochemical subtype and age) was calculated by the Wilcoxon-signed rank test.

Survival analyses

For survival analyses in each study, the Kaplan-Meier method (the log-rank test) was used, whereas the hazard ratios and 95% confidence intervals (CI) were calculated with the Cox proportional hazard regression model.

In the first retrospective study with NET, durations of progression-free survival (PFS) and overall survival (OS) times were calculated from day 1 of NET to the date of any tumor progression (local/regional progression, local relapse after surgery, or distant metastasis) and the date of death for any reason, respectively. Analyses were carried out from the aspects of the associations of the tumor response and the PFS with the initial tumor predictive markers, such as the ER, PgR and HER2 status, and the Ki67 and TOP2A protein expressions, the remaining tumor volume/risk group, and the differences in the tumor characteristics after the NET. The effects of the pathology results on PFS were analyzed with the Cox regression model.

Predictive role of the *LAPTM4B* copy number in the adjuvant cohort was investigated as follows. Distant metastasis free survival (DMFS) was assessed and defined as the time elapsed between the first diagnosis of primary breast carcinoma to the date of appearance of distant metastasis. The occurrence or absence of distant metastasis was considered as an indirect surrogate marker for response to different chemotherapy regimens. Receiver operating characteristic (ROC) curve analysis was used to determine the optimal cut-off value of *LAPTM4B* copy number used in survival tests. The Multivariate Cox regression analysis included the known breast carcinoma prognostic factors such as age at the initial diagnosis, pT, and pN status besides the *LAPTM4B* copy number.

For evaluation of the association between StrTIL and survival, the DMFS was assessed and defined as the time interval between the first cycle of pre-operative chemotherapy and the date of distant relapse or death. Nine cases with bone metastases at baseline were excluded from the DMFS analyses. The prognostic value of StrTIL changes, ΔStrTIL: the difference between post-StrTIL (surgical sample) and pre-StrTIL (core biopsy), was tested as continuous variable.

Multivariate Cox regression analysis included the following prognostic factors: age, grade, HR status, type of treatment, residual tumor size and post-treatment pathological lymph node status. The Kaplan–Meier method was used to analyze the role of ΔStrTIL in DMFS in HR negative and HR positive tumor groups separately.

4. Results

4.1 One-year neoadjuvant endocrine therapy in breast cancer

Therapeutic effects

Data of 42 patients were analyzed in the present analysis, four of them had bilateral breast cancers (n=46 tumors). The mean age of the cohort was 62 years (range: 36-82 years). 33 patients (78.6%; n=36 tumors) were postmenopausal (mean age: 67 years); 9 patients (21.4%; n=10 tumors) were premenopausal (mean age: 45 years).

The histologic type was invasive ductal carcinoma (n=35, 81.4%), invasive lobular carcinoma (n=7, 16.3%) or mucinous carcinoma (n=1, 2.3%). The patient- and tumor-related features are shown in Fig. 1.

All postmenopausal patients were treated with letrozole, while among the premenopausal patients, 7 and 2 patients received letrozole and tamoxifen, respectively, in combination with goserelin. In three patients (n=4 tumors, 8.7%), the hormone therapy was changed to chemotherapy because of local progression. These patients were included in the survival analyses, but the pathological results on their surgical specimens were not used. In two postmenopausal patients, letrozole was replaced by tamoxifen because of the insufficient treatment effect after 6 or 9 months. After an overall 12 months of NET, these patients exhibited partial regression on clinical examination, but no relevant regression at pathological evaluation; mastectomy was performed in both cases. The imaging responses to NET were as follows: complete response (CR): 7/46 (15.2%); partial response (PR): 30/46 (65.2%); no change: 5/46 (10.9%); progression: 4/46 (8.7%). The following surgical interventions were carried out: excision n=19/42 (45.2%), mastectomy: 23/42 (54.8%); sentinel lymph node biopsy (SNB): 20/42 (47.6%); axillary block dissection±SNB: 22/42 (52.4%).

According to the histopathological examination, all the 42 assessable tumors showed pathological response to NET. Best response, pCR was observed in 14.3% (6/42) of the tumors (6/46, 13.0% of all tumors). In four cases, there was residual cancer neither in the breast nor in the lymph nodes while in two cases, only DCIS remained (risk group 1). Most cases (25/46, 54.3%) belonged to risk group 2, in two patients invasive residual carcinoma was detected only in the lymph nodes, and in one node-negative patient, only isolated tumor cells were found in the breast. Finally, risk group 3 comprised 11/46 (24.0%) cases with stage IIB≤ residual tumor and 4/46 (8.7%) cases with clinical progression. The treatment results are presented in Fig. 1.

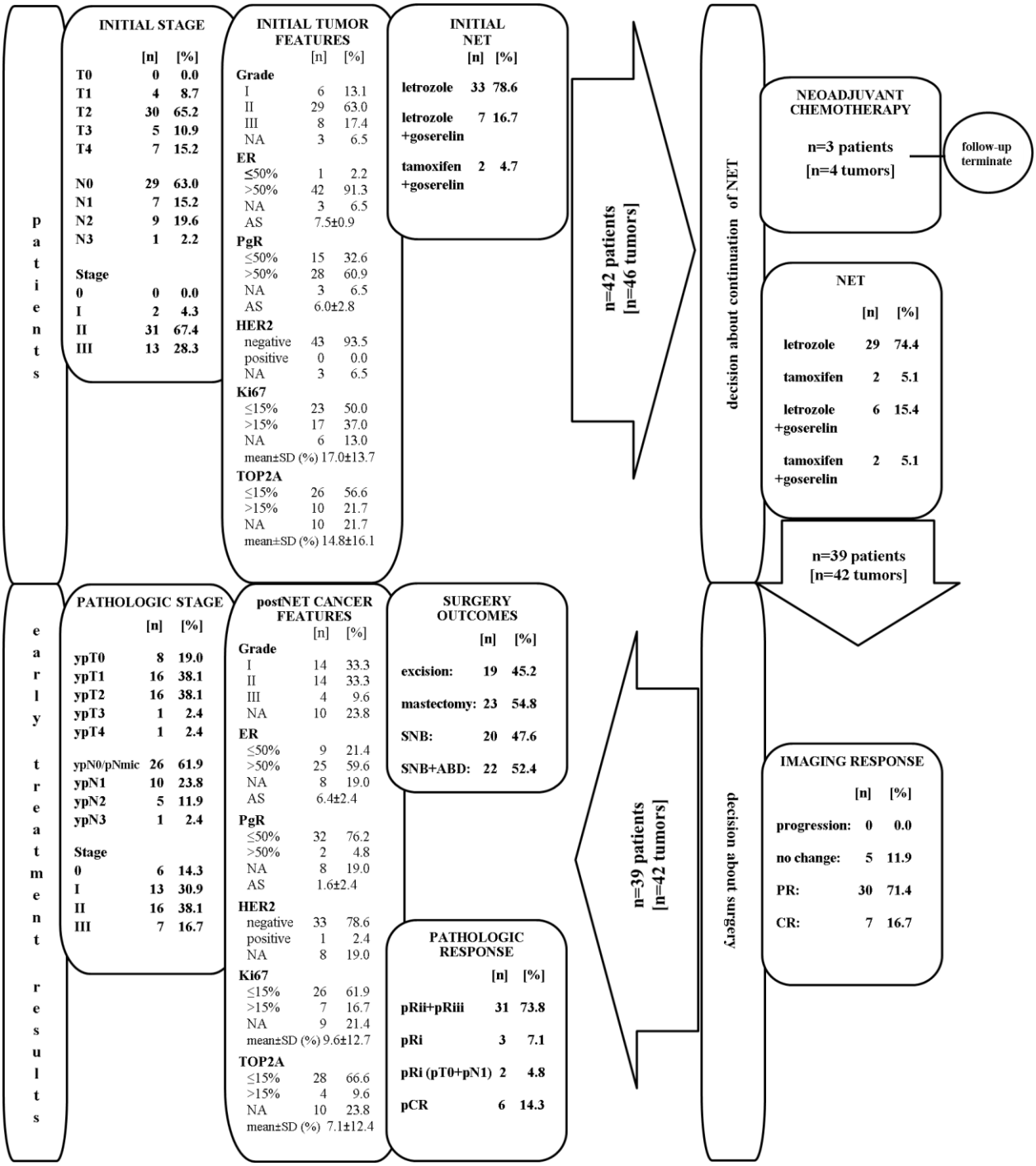


Figure 1 Overview of clinical events and therapy responses during neoadjuvant endocrine treatment

Five of 39 patients received adjuvant chemotherapy after the surgery due to the lack of a significant therapeutic effect, and in one case because of the change in the phenotype to triple negativity. Altogether 37/39 patients continued the same endocrine therapy as that before the surgery.

Most tumors in the premenopausal group (9/10, 90.0%) were of stage II and grade 1-2, the expression of ER and PgR was similarly high, while that of Ki67 and TOP2A was less than in postmenopausal patients (Fig. 2); 2/10 tumors showed pCR. Altogether 4 premenopausal patients received chemotherapy, 1 patient before and 3 patients after the surgery.

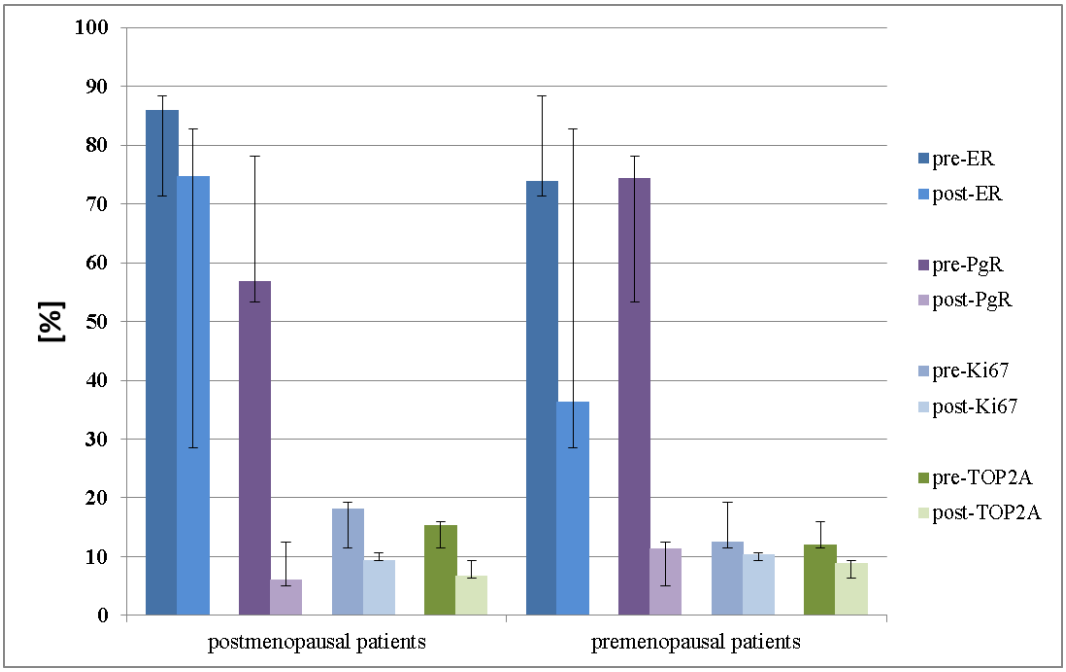


Figure 2 ER, PgR, Ki67 and TOP2A expression among postmenopausal and premenopausal cases before and after NET

Association between tumor response to NET and tumor characteristics

A higher initial ER expression was related to a better response to NET (Table 1). The likeliness of a good response to NET was increased by 7% for every 1% increase of the expression of ER (odds ratio: 1.070; CI: 1.007–1.138, p=0.029). No significant associations were detected between the initial tumor grade or the expression of PgR, Ki67 or TOP2A and the therapeutic response (Table 1).

The changes in ER, PgR or HER2 expression after NET were analyzed in 32 tumors since the cases that progressed (n=4) or in which there was no remaining invasive tumor in the breast (n=8) could not be included (Fig. 1). The average expression (±SD) of ER (85.2±15.1% *vs.* 65.4±32.9%; p=0.002), PgR (66.1±32.3% *vs.* 7.7±17.7%; p=0.001), Ki67 (17.9±12.2% *vs.* 10.1±13.0%; p=0.012) and TOP2A (16.8±17.8 *vs.* 7.4±12.8; p=0.029) decreased significantly in the surgical specimens as compared with the core biopsies taken before the treatment. In one patient, the tumor completely lost both ER and PgR expression after NET. The HER2 status did not display significant changes, however in a single case, although the core biopsy

indicated HER2 negativity, in the surgical specimen, IHC showed HER2 positivity, and FISH revealed the gene amplification.

Table 1 Clinical and initial pathological characteristics of the three risk groups

	Group 1 n=6 (5 patients)	**Group 2** n=25 (23 patients)	**Group 3** n=15 (14 patients)	**p**
mean age (years, ±SD)	61.4±14.3	65.8±10.0	56.7±13.1	0.080
menopausal status				
premenopausal [n=9 patients] (n, %)	1 (11.1)	2 (22.2)	6 (66.7)	0.040
postmenopausal [n=33 patients] (n, %)	4 (12.1)	21 (63.6)	8 (24.2)	
grade [n=43 tumors]				
I (n, %)	2 (33.3)	1 (16.7)	3 (50.0)	0.134
II (n, %)	2 (6.9)	16 (55.2)	11 (37.9)	
III (n, %)	1 (12.5)	6 (75.0)	1 (12.5)	
ER [n=43 tumors]				
percentage of stained tumor cells (mean±SD)	84.0±8.9	88.3±9.4	75.7±19.9	0.034
Allred score (mean±SD)	7.4±0.9	7.8±0.6	7.2±1.1	0.112
PgR [n=43 tumors]				
percentage of stained tumor cells (mean±SD)	48.0±34.2	68.7±29.3	52.3±40.7	0.252
Allred score (mean±SD)	4.8±2.9	6.9±2.0	5.1±5.1	0.106
Ki67 [n=40 tumors]				
percentage of stained tumor cells (mean±SD)	23.0±23.1	17.7±11.4	13.3±13.5	0.399
≤15% (n, %)	3 (50.0)	11 (44.0)	9 (60.0)	0.302
>15% (n, %)	2 (33.3)	12 (48.0)	3 (20.0)	
TOP2A [n=36 tumors]				
percentage of stained tumor cells (mean±SD)	13.0±6.7	15.6±16.4	14.1±19.7	0.939
≤15% (n, %)	3 (50.0)	15 (60.0)	8 (53.3)	0.712
>15% (n, %)	2 (33.3)	6 (24.0)	2 (13.3)	

Survival after NET

After a median follow-up time of 45.2 (range: 17.2-111.6) months, six patients developed distant metastases, and one patient had a second metachronous cancer in the opposite breast. Three patients died, two because of metastatic breast cancer, and one for a reason other than breast cancer. The estimated mean PFS time was 74.2 (CI: 60.4-88.0) months and the estimated mean OS time was 92.8 (CI: 80.0–105.7) months. The tumor volume remaining after NET predicted PFS levels of 85.3, 70.6 and 41.4 months in risk groups 1, 2 and 3, respectively (p=0.001) (Fig. 3a). The hazard ratio for PFS in groups 1 and 2 was 0.131 (CI: 0.016-1.056, p=0.056) and 0.101 (CI: 0.022-0.468, p=0.003), respectively, as compared with group 3. No significant associations were detected between the pre-treatment or post-treatment ER, PgR and TOP2A statuses or their changes and the PFS. High expression (>15%) of Ki67 in the surgical specimen predicted a risk of progression (hazard ratio: 5.432, CI: 1.202-24.553, p=0.028). OS was not analyzed in relation with these parameters because of the limited number of events.

As a result of extended follow-up (median 61.0 months, range: 17.8-136.4 months) of all patients, the estimated mean PFS was 93.0 months (CI: 77.0-108.9). During this period, metastases developed in 5 additional cases, hence overall 11/42 patients had distant metastases (1/5 in risk group 1; 4/23 in risk group 2; 6/14 in risk group 3); one additional patient had a contralateral breast cancer. Overall 13 patients died, 8 of them due to breast carcinoma, whereas 5 patients deceased of another cause. According to the menopausal status, event occurred in 3/9 premenopausal (33.3%) and 8/33 postmenopausal women (24.2%). Although, there was no significant difference in PFS between the premenopausal and postmenopausal cases (p=0.257), the estimated mean PFS was 61.5 months (CI: 38.1-84.9) and 101.5 months (CI: 83.6-119.4), respectively.

Among those 34 patients who had been treated with sole endocrine therapy, 26 remained breast cancer-free.

Among the patients who received additional neoadjuvant (n=3) or adjuvant (n=5) chemotherapy, three cases had distant metastases and in one patient developed second breast cancer.

Updated survival analysis showed similar survival outcome in the 3 risk groups as earlier. There was no difference in PFS between risk group 1 and 2 (p=0.618), while the risk group 3 showed the worst outcome (estimated mean PFS, CI: 113.9 months, 92.2-135.7; 97.5 months, 83.2-111.8; 50.9 months, 24.7-77.1; p<0.001; Fig. 3b).

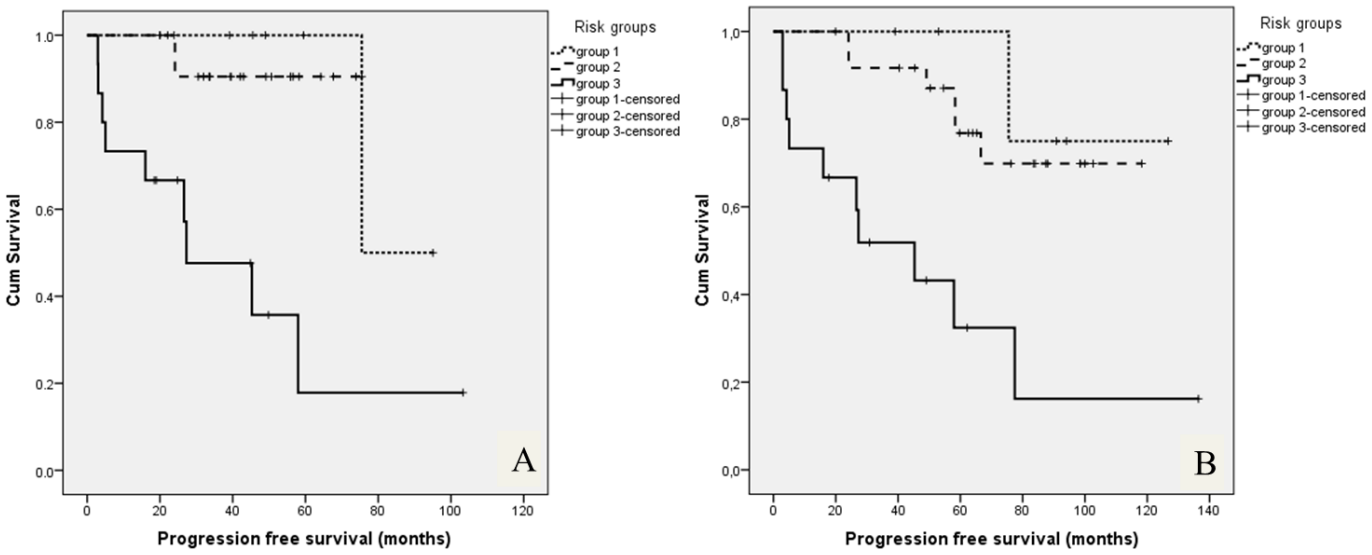


Figure 3 Progression-free survival in the three risk groups after the completion of neoadjuvant endocrine therapy as estimated by the Kaplan-Meier method (n=42 patients); median follow-up time: 45.2 months, p=0.001 (**a**) extended median follow-up time: 61.0 months p<0.001 (**b**)

4.2 LAPTM4B gene copy number gain and response to anthracycline-based chemotherapy in hormone receptor negative breast carcinomas

A total of 143 cases with HR-negative breast cancer were enrolled into two cohorts. The first cohort included 69 core biopsies of HR-negative (64/69 TNBC and 5/69 HER2-positive) primary breast carcinoma cases. The mean age of patients was 50 years (range: 26-79 years) all cases had invasive carcinoma of no special type. After neoadjuvant therapy, pCR was achieved in 26 cases (37.7%), pPR in 38 cases (55.1%), and pNR in 5 cases (7.2%). The clinicopathological data are presented in Table 2. The average *LAPTM4B*/CEN8q ratio was ≥2.0 in only 6/69 (8.6%) cases with the highest ratio being 3.71.

Considering the average *LAPTM4B* copy number/cell in the group of patients receiving anthracycline-based neoadjuvant therapy, higher average *LAPTM4B* gene copy number was observed in the pNR group compared to pCR group (4.1±1.1 *vs.* 2.6±0.1, p=0.029) (Figs. 4a-b, 5a). We also compared average *LAPTM4B* gene copy numbers between patients who had no regression or who presented minimal response to anthracycline-based neoadjuvant therapy (>50% residual tumor remaining) (pNR+pPRiii) *versus* cases with pCR. Again, significantly higher average gene copy number was found in the group of patients with inferior response to anthracycline-based neoadjuvant therapy (3.3±0.3 *vs.* 2.6 ± 0.1, p=0.035, Fig. 5c).

The same is true for average CEN8q being significantly higher in the pNR and pNR+pPRiii groups compared to pCR group (3.7±0.9 *vs.* 2.2±0.1, p=0.048 and 2.9±0.3 *vs.* 2.2±0.1, p=0.040 respectively).

In the non-anthracycline-treated group of patients, we observed pNR in a single case (Fig. 5b). Therefore, we compared *LAPTM4B* gene copy number between pNR+pPRiii and pCR groups, resulting no significant difference (p=0.360) (Fig. 5d).

Regarding average CEN8q copies, in the non-anthracycline-treated group of patients, no significant differences were observed between pNR+pPRiii and pCR groups (p=0.879).

Table 2 Clinicopathological characteristics of patients treated with neoadjuvant anthracycline-based *vs.* non-anthracycline-based chemotherapy

Parameter	**Anthracycline-based group** (n=50)	**Non-anthracycline-based group** (n=19)
mean age (years)	50 (range: 26-79)	52 (range: 29-76)
histological grade		
grade 2	6 (12.0%)	1 (5.3%)
grade 3	44 (88.0%)	18 (94.7%)
IHC-based molecular types		
TNBC	46 (92.0%)	18 (94.7%)
HER2-positive	4 (8.0%)	1 (5.3%)
clinical tumor stage		
primary tumor		
T1	1 (2.0%)	1 (5.3%)
T2	29 (58.0%)	13 (68.4%)
T3	10 (20.0%)	2 (10.5%)
T4	10 (20.0%)	3 (15.8%)
regional lymph node		
N0	15 (30.0%)	10 (52.6%)
N1	13 (26.0%)	6 (31.6%)
N2	16 (32.0%)	3 (15.8%)
N3	6 (12.0%)	0 (0.0%)
pathological response to neoadjuvant therapy		
pCR	16 (32.0%)	10 (52.6%)
pPR	30 (60.0%)	8 (42.1%)
pPRi	6 (20%)	0 (0%)
pPRii	12 (40%)	1 (12.5%)
pPRiii	12 (40%)	7 (87.5%)
pNR	4 (8.0%)	1 (5.3%)
chemotherapy regimens		
TE/TA/TAX+E/TAX+A/TAC	20 (40.0%)	-
TEX/FEC+T	25 (50.0%)	-
FEC	5 (10.0%)	-
T+CDDP	-	12 (63.1%)
T+CBP/TAX+CBP	-	7 (36.9%)
neoadjuvant trastuzumab	1 (2.3%)	1 (5.9%)
average cycle no.	5.5 (range: 2-6)	5.5 (range: 2-6)

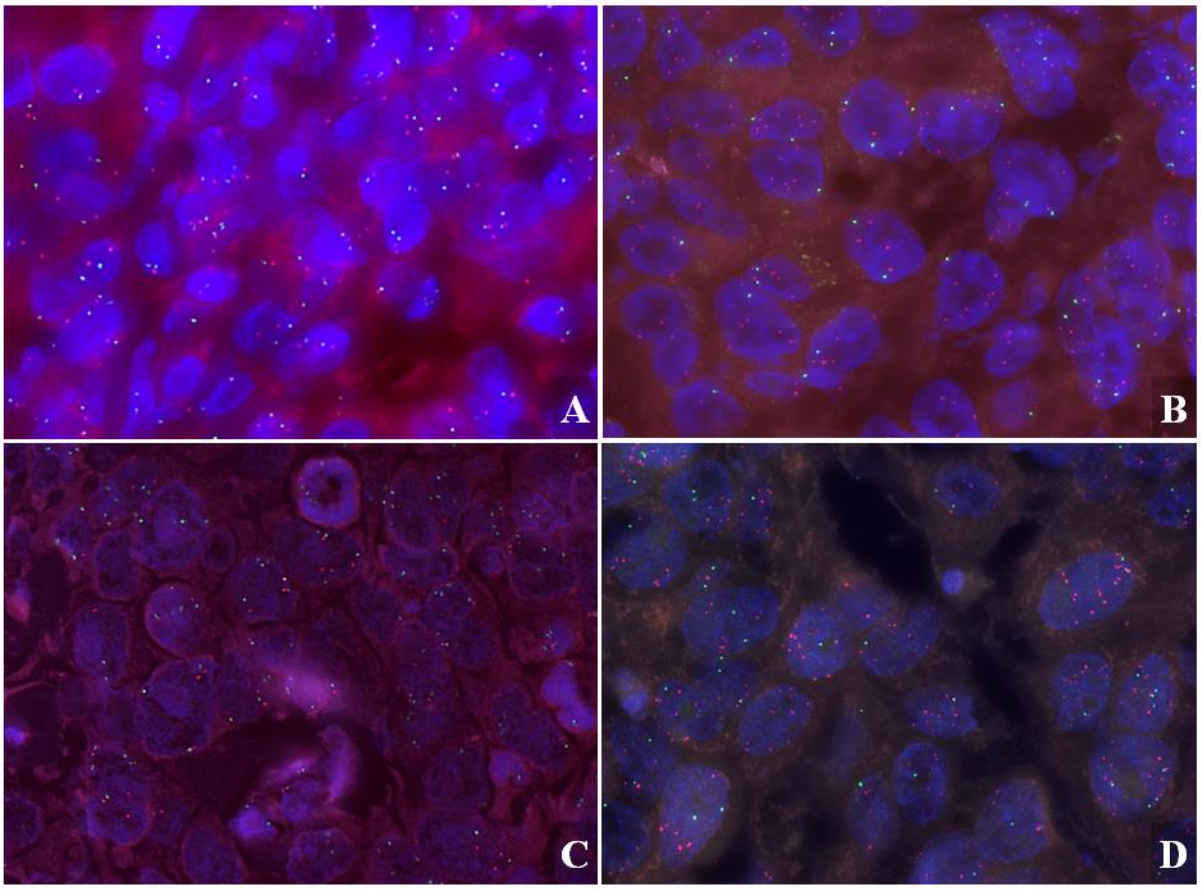


Figure 4 *LAPTM4B* FISH images in anthracycline-treated cases.
Normal *LAPTM4B* copy number in a core biopsy of case with pCR after neoadjuvant therapy (**a**) and increased average *LAPTM4B* gene copy number in a case without any therapy response after neoadjuvant therapy (**b**); a primary breast carcinoma case treated with adjuvant chemotherapy and presenting no distant organ metastasis during the follow-up period (**c**) and a primary breast carcinoma case who had increased average *LAPTM4B* gene copies and recurrence in distant organs later on (**d**).
LAPTM4B gene was labelled with red, whereas chromosome 8 centromeric region was stained with green fluorescent dye. Cell nuclei were counterstained with DAPI (blue). FISH photos were acquired using 63×objective.

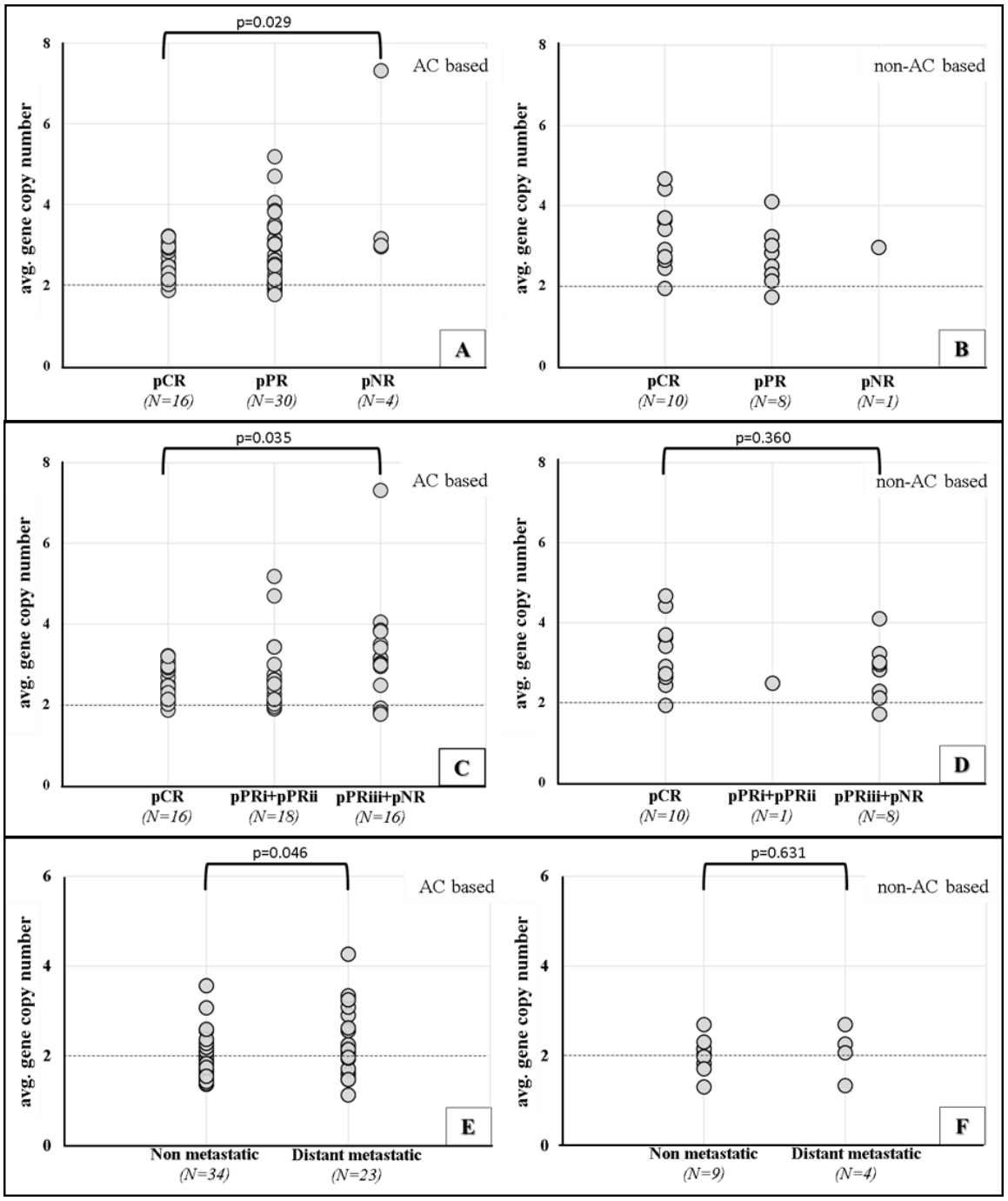


Figure 5 Association between average *LAPTM4B* copy number and therapy response in the neoadjuvant cohort (**a-d**) and in the adjuvant cohort (**e, f**).
In the neoadjuvant anthracycline-treated cases, significantly higher average *LAPTM4B* gene copy number was observed in the pNR group compared to the pCR group (p=0.029) (**a**). In the neoadjuvant non-anthracycline-treated cases, we observed pNR in a single patient (**b**). Significantly higher average *LAPTM4B* gene copy number was observed in the pNR+pPRiii group as compared to the pCR group (p=0.035) among neoadjuvant anthracycline-treated cases (**c**), whereas, in the non-anthracycline-treated cases, no significant differences were observed between the two groups (**d**). Average gene copy number was significantly higher in metastatic cases as compared to the non-metastatic cases in adjuvant anthracycline-treated cases (p=0.046) (**e**) and no significant differences in the non-anthracycline-treated cases (**f**). A $p \leq 0.050$ was considered statistically significant using two-sided Mann-Whitney-Wilcoxon exact test. AC based: anthracycline-treated; non-AC based: non-anthracycline-treated

In the adjuvant treated cohort, 74 FFPE samples of surgically removed HR-negative breast carcinomas (39/74 TNBC, 27/74 HER2-positive and 8/74 with no reliable HER2 data) were collected, the mean age of the patients was 52 years (32-81).

Most cases (94.6%) had invasive carcinoma of no special type and 5.4% presented other histological type (n=1 invasive lobular carcinoma, n=1 carcinoma anaplasticum, n=1 carcinoma medullare, n=1 apocrin carcinoma). During the follow-up period, distant metastases occurred in 30 (40.5%) cases. The clinicopathological data are presented in Table 3.

LAPTM4B/CEN8q ratio ≥2.0 was observed in only 4/74 (5.4%) cases. Again considering the average *LAPTM4B* gene copy number in the adjuvant anthracycline-treated patient cohort, the average *LAPTM4B* gene copy number was higher in metastatic cases, compared to the non-metastatic ones (2.2±0.2 *vs.* 1.9±0.1, p=0.046, Figs. 4c-d, 5e). In patients treated with other than anthracycline chemotherapy, no significant differences were detected between metastatic *vs.* non-metastatic groups (Fig. 5f).

Regarding average CEN8q copies, no significant differences were observed between metastatic *vs.* non-metastatic groups neither in anthracycline-treated nor in non-anthracycline-treated patients.

Comparison of the two HR-negative subtypes (HER2-positive and TNBC cases) showed no significant differences in the average *LAPTM4B* gene copy number/cell (p=0.328). Kaplan-Meier curve estimation based on DMFS revealed that higher *LAPTM4B* copy number was an independent predictor for DMFS in the anthracycline-treated adjuvant cohort (log-rank test, p=0.037). Cut-off value for poor prognosis was defined as follows: the ratio of amplified cell population (*LAPTM4B*/CEN8q≥2.0) is more than 15% and the average gene copy number is more than 2.5 per sample (Fig. 6). Based on these criteria, of the 22/57 patients treated with anthracycline-based adjuvant chemotherapy and diagnosed with distant metastases, 6/22 cases presented higher *LAPTM4B* gene copy number, whereas, in 16/22 cases, lower *LAPTM4B* gene copy number was detected. Cox regression analysis was also performed, revealing association between increased *LAPTM4B* gene copy number and worse DMFS (p=0.044).

Table 3 Clinicopathological characteristics of cases treated with adjuvant anthracycline-based *vs.* non-anthracycline-based chemotherapies

Parameter	**Anthracycline-based group** (n=57)	**Non-anthracycline-based group** (n=13)
mean age (years)	53 (range: 32-76)	50 (range: 32-81)
histological grade		
grade 1	2 (3.5%)	1 (7.7%)
grade 2	14 (24.6%)	4 (30.8%)
grade 3	39 (68.4%)	8 (61.5%)
unknown	2 (3.5%)	0 (0.0%)
molecular type based on IHC		
TNBC	28 (49.1%)	10 (76.9%)
HER2-positive	22 (38.6%)	2 (15.4%)
HR-negative, HER2 n.a.	7 (12.3%)	1 (7.7%)
pathologic tumor stage		
primary tumor		
pT1	16 (28.1%)	1 (7.7%)
pT2	30 (52.6%)	7 (53.8%)
pT3	5 (8.8%)	2 (15.4%)
pT4	4 (7.0%)	1 (7.7%)
Unknown	2 (3.5%)	2 (15.4%)
regional lymph node		
pN0	13 (22.8%)	5 (38.5%)
pN1	18 (31.6%)	2 (15.4%)
pN2	14 (24.6%)	1 (7.7%)
pN3	2 (3.5%)	2 (15.4%)
Unknown	10 (17.5%)	3 (23.0%)
distant metastasis		
yes	23 (40.4%)	4 (30.8%)
no	34 (59.6%)	9 (69.2%)
chemotherapy regimen*		
AC/EC	19 (33.3%)	-
AC+CMF/EC+CMF/FAC/FEC	25 (43.9%)	-
TAC/AC+T/EC+T/AC+TAX	11 (19.3%)	-
FEC+T /FAC+T	2 (3.5%)	-
Methotrexate	-	2 (15.4%)
CMF	-	9 (69.2%)
T	-	1 (7.7%)
CBP+T	-	1 (7.7%)
trastuzumab added	5 (8.9%)	1 (7.7%)

*unknown n=4

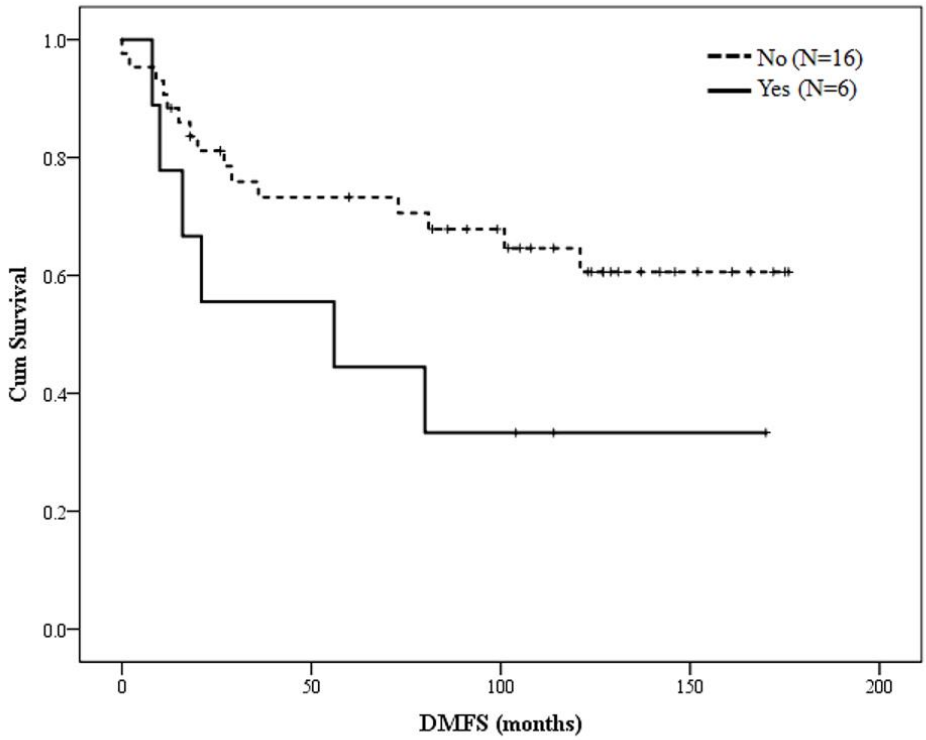


Figure 6 Kaplan-Meier curve estimation of DMFS in the anthracycline-treated adjuvant cohort.
Cut-off value for poor prognosis was defined as follows: the ratio of amplified cell (*LAPTM4B*/CEN8q≥2.0) population is more than 15% and the average gene copy number is more than 2.5 per sample. Based on this criterion, 6/22 cases presented higher *LAPTM4B* gene copy number, whereas, in 16/22 cases, lower *LAPTM4B* gene copy number was detected (log rank test, p value was 0.037)

4.3 Influence of mutagenic versus non-mutagenic pre-operative chemotherapy on the immune infiltration of breast cancer

Samples from 112 individuals were available for analysis. The majority of patients (n=103) received neoadjuvant chemotherapy plus surgery with curative intent, while 9 women had bone metastases already at the beginning of the pre-operative chemotherapy. Most patients (86.6%, n=97) had invasive carcinoma of no special type, 42.0% (n=47) were HR-negative and 58.0% (n=65) were HR-positive. The clinicopathological characteristics are reported in Table 4. At initiation of pre-operative chemotherapy the mean age of the patients was 55 years (range: 29-80 years). A quarter of the patients (n=28) received platinum-based therapy, 37.5% (n=42) received cyclophosphamide-based therapy and 37.5% (n=42) received anthracycline-based therapy.

Of the 28 patients undergoing platinum-based therapy, 64.3% (n=18) were HR-negative (mostly triple-negative, 46.4%, n=13), while 35.7% (n=10) were HR-positive.

Out of the 42 patients undergoing cyclophosphamide-based therapy, 23.8% (n=10) were HR-negative and 76.2% (n=32) were HR-positive (Table 4).

Of the 42 patients undergoing anthracycline-based therapy, 45.2% (n=19) had HR-negative and 54.8% (n=23) had HR-positive carcinomas.

The majority of patients received more than four cycles of chemotherapy and the average cycle number was similar among the groups (Table 4).

Out of the 22 HER2-positive cases, 68.2% (n=15) received pre-operative trastuzumab therapy. Trastuzumab was administered in combination with platinum-based (n=8), cyclophosphamide-based (n=5) or anthracycline-based (n=2) therapy (Table 4).

Table 4 Clinico-pathological characteristics of patients in the study on stromal tumor-infiltrating lymphocytes

	All, n=112 (%)	Platinum-based group, n=28 (%)	Cyclophosphamide-based group, n=42 (%)	Anthracycline-based group, n=42 (%)
mean age (years; range)	55 (29-80)	53 (29-80)	55 (35-79)	57 (32-78)
histological type (core biopsy)				
invasive carcinoma NST	97 (86.6)	23 (82.1)	41 (97.6)	33 (78.6)
ILC	12 (10.7)	4 (14.3)	1 (2.4)	7 (16.6)
other	3 (2.7)	1 (3.6)	0 (0.0)	2 (4.8)
immunohistochemical type (core biopsy)				
HR-positive	65 (58.0)	10 (35.7)	32 (76.2)	23 (54.8)
HR-positive/HER2-negative	53 (47.3)	4 (14.3)	27 (64.3)	22 (52.4)
HR-positive/HER2-positive	12 (10.7)	6 (21.4)	5 (11.9)	1 (2.4)
HR-negative	47 (42.0)	18 (64.3)	10 (23.8)	19 (45.2)
HR-negative/HER2-positive	10 (8.9)	5 (17.9)	1 (2.4)	4 (9.5)
triple-negative	37 (33.1)	13 (46.4)	9 (21.4)	15 (35.7)
histological grade (core biopsy)				
grade 1	3 (2.7)	0 (0.0)	1 (2.4)	2 (4.8)
grade 2	40 (35.7)	8 (28.6)	16 (38.1)	16 (38.1)
grade 3	66 (58.9)	19 (67.8)	23 (54.8)	24 (57.1)
unknown	3 (2.7)	1 (3.6)	2 (4.8)	0 (0.0)
HR-positive				
grade 1	3 (2.7)	0 (0.0)	1 (2.4)	2 (4.8)
grade 2	31 (27.7)	3 (10.7)	15 (35.7)	13 (31.0)
grade 3	29 (25.9)	7 (25.0)	14 (33.3)	8 (19.0)
unknown	2 (1.8)	0 (0.0)	2 (4.8)	0 (0.0)
HR-negative				
grade 1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
grade 2	9 (8.0)	5 (17.9)	1 (2.4)	3 (7.1)
grade 3	37 (33.0)	12 (42.8)	9 (21.4)	16 (38.1)
unknown	1 (0.9)	1 (3.6)	0 (0.0)	0 (0.0)
average number of pre-operative chemotherapy cycles (range)	5.3 (2-8)	5.1 (2-8)	5.1 (2-6)	5.5 (3-8)
number of pre-operative chemotherapy cycles				
≤4	34 (30.4)	9 (32.1)	15 (35.7)	10 (23.8)
>4	78 (69.6)	19 (67.9)	27 (64.3)	32 (76.2)
chemotherapy regimens				
carboplatin+docetaxel or paclitaxel	21 (18.8)	21 (75.0)	-	-
cisplatin+docetaxel or paclitaxel	7 (6.3)	7 (25.0)	-	-
AC	2 (1.8)	-	2 (4.8)	-
FEC	15 (13.4)	-	15 (35.7)	-
CMF	1 (0.9)	-	1 (2.4)	-
AC+docetaxel or FEC+docetaxel	24 (21.4)	-	24 (57.1)	-
epirubicin+docetaxel or paclitaxel	32 (28.5)	-	-	32 (76.2)
doxorubicin+docetaxel or paclitaxel	10 (8.9)	-	-	10 (23.8)
pre-operative trastuzumab	15 (13.4)	8 (28.6)	5 (11.9)	2 (4.8)
ypT				
<2cm	42 (37.5)	11 (39.3)	17 (40.5)	14 (33.3)
≥2cm	69 (61.6)	16 (57.1)	25 (59.5)	28 (66.7)
unknown	1 (0.9)	1 (3.6)	0 (0.0)	0 (0.0)
ypN				
negative	31 (27.7)	9 (32.1)	11 (26.2)	11 (26.2)
positive	75 (67.0)	18 (64.3)	29 (69.0)	28 (66.7)
unknown	6 (5.3)	1 (3.6)	2 (4.8)	3 (7.1)
ΔStrTIL				
zero or positive	87 (77.7)	22 (78.6)	35 (83.3)	30 (71.4)
negative	25 (22.3)	6 (21.4)	7 (16.7)	12 (28.6)

StrTIL changes before and after chemotherapy

In the pre-treatment core biopsy samples, the median pre-StrTIL was 3.00% (interquartile range (IQR): 1.00-7.50) and more than 50% StrTIL (lymphocyte predominant) was detected in only one case. The post-StrTIL reached 50% or above in 10 cases (the pre-operative therapy was platinum-based (n=4), FEC (n=1) or docetaxel plus epirubicin (n=5), (Figs. 7a-i).

The median post-StrTIL rose significantly to 6.25% (IQR: 3.00-25.00; $p<0.001$) after treatment. Pre-StrTIL less than 1% was observed in 14 cases, while StrTIL less than 1% in the residual tumor occurred in only two cases.

The increase in post-StrTIL was significant both in HR-positive (ΔStrTIL positive: n=32 (49.2%); zero: n=21 (32.3%); negative: 12 (18.5%) and HR-negative (ΔStrTIL positive: n=29 (61.7%); zero: n=5 (10.6%); negative: n=13 (27.7%) cases ($p<0.001$ in both groups; Table 5).

In the subgroup of HR-positive/HER2-negative cases, the changes in StrTIL was significant in grade 3 cases (ΔStrTIL positive: n=14 (66.7%); zero: n=3 (14.3%); negative: n=4 (19.0%); $p=0.007$) but not in grade 1-2 cases (ΔStrTIL positive: n=11 (36.6%); zero: n=14 (46.7%); negative: n=5 (16.7%); $p=0.075$; Table 5).

We did not detect any association between changes in StrTIL and other features (shown in Table 5).

When analyzing the pre-StrTIL and post-StrTIL among the three treatment groups, we experienced significant StrTIL increase independently from the treatment applied (Tables 5-6; Figs. 7a, 7d, 7g). Interestingly, in the subgroup analysis, only the administration of cyclophosphamide resulted in a significant increase in StrTIL in HR-positive cases (ΔStrTIL positive: n=18 (56.3%); zero: n=10 (31.2%); negative: n=4 (12.5%); $p<0.001$; Tables 5-6; Figs. 7c, 7f, 7i).

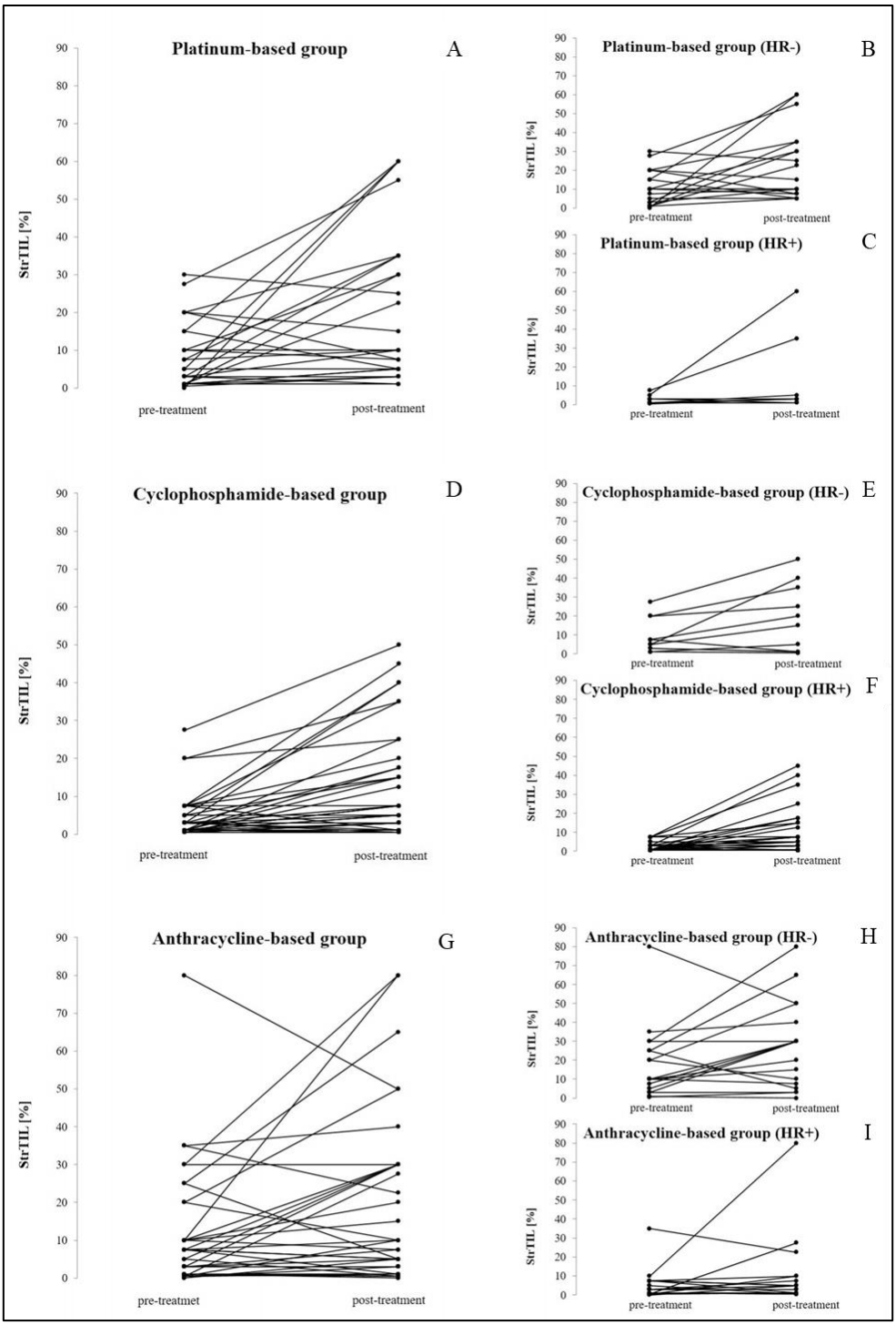


Figure 7 Stromal tumor-infiltrating lymphocytes (StrTIL) before and after pre-operative chemotherapy

Significant ΔStrTIL increase was observed in the three treatment groups (platinum-based: p=0.007 (**a**); cyclophosphamide-based: p<0.001 (**d**); anthracycline-based: p=0.047 (**g**). By analyzing separately the HR-positive (**c**, **f**, **i**) and HR-negative cases, only the administration of cyclophosphamide resulted in significant ΔStrTIL increment in HR-positive cases (p<0.001 (**i**), whereas in HR-negative cases, no strong relationship between the treatment applied and StrTIL changes could be proven (platinum-based: p=0.026 (**b**); cyclophosphamide-based: p=0.049 (**e**); anthracycline-based: p=0.063 (**h**).

Table 5 Changes in stromal tumor-infiltrating lymphocytes (StrTIL): median StrTIL levels before and after pre-operative chemotherapy

		n	pre-StrTIL; median [%]; (IQR)	post-StrTIL; median [%]; (IQR)	p value (Wilcoxon Signed-test)
whole population		112	3.00 (1.00-7.50)	6.25 (3.00-25.00)	***<0.001***
age	<50	35	3.00 (1.00-7.50)	5.00 (1.00-25.00)	***0.001***
	≥50	77	3.00 (1.00-8.75)	7.50 (3.00-26.25)	***<0.001***
grade	1-2	43	1.00 (1.00-3.00)	3.00 (1.00-7.50)	***0.011***
	3	66	5.00 (1.00-11.25)	15.00 (3.00-35.00)	***<0.001***
HR-positive		65	1.00 (1.00-3.00)	3.00 (1.00-8.75)	***<0.001***
	HER2-negative	53	1.00 (1.00-3.00)	3.00 (1.00-7.50)	***0.002***
	HER2-positive	12	2.00 (1.00-3.00)	4.00 (1.50-16.88)	***0.020***
HR-negative		47	10.00 (3.00-20.00)	20.00 (5.00-35.00)	***<0.001***
	HER2-positive	10	3.00 (0.88-13.1)	27.5 (8.75-32.50)	***0.012***
	triple-negative	37	10.00 (5.00-20.00)	20.00 (5.00-35.00)	***0.008***
HR-positive/HER2-negative	grade 1-2	30	1.0 (1.00-3.00)	3.0 (1.00-5.62)	0.075
	grade 3	21	1.00 (1.00-6.25)	5.0 (1.00-16.25)	***0.007***
platinum-based group		28	4.00 (1.00-13.75)	10.00 (3.00-33.75)	***0.007***
	HR-positive	10	2.00 (1.00-3.50)	3.00 (1.00-12.50)	0.094
	HR-negative	18	10.00 (2.50-20.00)	18.75 (7.50-35.00)	***0.026***
cyclophosphamide-based group		42	1.00 (1.00-5.00)	5.00 (1.00-17.50)	***<0.001***
	HR-positive	32	1.00 (1.00-3.00)	4.00 (1.00-14.38)	***<0.001***
	HR-negative	10	6.25 (2.50-20.00)	17.50 (1.00-36.25)	***0.049***
anthracycline-based group		42	4.00 (1.00-10.00)	5.00 (2.50-30.00)	***0.047***
	HR-positive	23	3.00 (1.00-7.50)	3.00 (1.00-7.50)	0.502
	HR-negative	19	10.00 (3.00-25.00)	30.00 (5.00-40.00)	0.063
cycle number	≤4	34	2.00 (1.00-5.63)	4.00 (1.00-18.13)	***<0.001***
	>4	78	3.00 (1.00-10.00)	7.50 (3.00-28.13)	***<0.001***
HER2-positive cases received pre-operative trastuzumab	yes	15	1.00 (1.00-3.00)	7.50 (3.00-25.00)	***0.006***
	no	7	5.00 (1.00-7.50)	30.00 (3.00-35.00)	***0.031***

Table 6 Changes of stromal tumor-infiltrating lymphocytes according to treatment

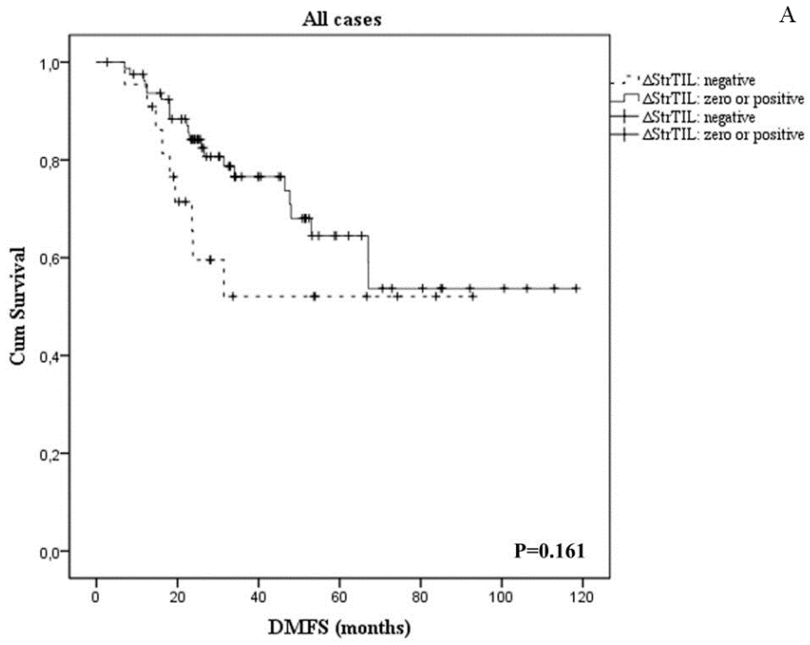
ΔStrTIL	Platinum-based			Cyclophosphamide-based			Anthracycline-based		
	all	HR negative	HR positive	**all**	HR negative	HR positive	**all**	HR negative	HR positive
	n (%)	n (%)	n (%)	**n (%)**	n (%)	n (%)	**n (%)**	n (%)	n (%)
positive	**16**	11	5	**25**	7	18	**20**	11	9
	(57.2)	(61.1)	(50.0)	**(59.5)**	(70.0)	(56.3)	**(47.6)**	(57.9)	(39.2)
zero	**6**	2	4	**10**	0	10	**10**	3	7
	(21.4)	(11.1)	(40.0)	**(23.8)**	(0.0)	(31.2)	**(23.8)**	(15.8)	(30.4)
negative	**6**	5	1	**7**	3	4	**12**	5	7
	(21.4)	(27.8)	(10.0)	**(16.7)**	(30.0)	(12.5)	**(28.6)**	(26.3)	(30.4)

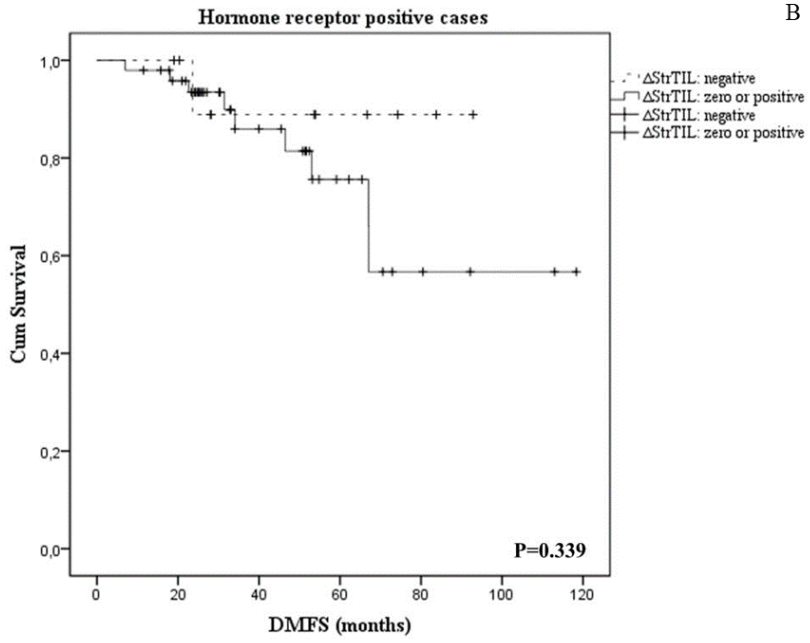
Survival analysis according to StrTIL status

Data on DMFS was available in 103 cases. The median DMFS was 28.2 months (range: 2.6-118.3 months). Distant metastases occurred in 31/103 (30.1%) cases. In 21/31 (67.7%) cases, the primary breast carcinoma was HR-negative, and in 19/31 (61.3%) cases the post-StrTIL was lower than 10.0% or showed a decrease in comparison with the pre-StrTIL value. As reported in Table 7, in univariate analyses, the HR status and the post-treatment pathological lymph node status were the only significant factors influencing DMFS. In the multivariate model changes of StrTIL showed a strong prognostic value (Table 7). The Cox analysis in HR-negative cases confirmed both post-StrTIL and ΔStrTIL as playing independent prognostic role in DMFS. Each 1% increase in post-StrTIL reduced the hazard of distant metastasis development by 2.6% (hazard ratio: 0.974; CI: 0.948-1.000; p=0.05) and for each 1% ΔStrTIL increment, the risk of distant metastasis was reduced by 4.3% (hazard ratio: 0.957; CI: 0932-0.983; p=0.001), but according to our results, the pre-StrTIL did not influence the DMFS. The prognostic role of StrTIL in HR-positive cases could not be proven (Table 8). The Kaplan-Meier analysis was carried out in HR-negative and HR-positive cases separately. Among HR-negative cases, increased or unchanged post-StrTIL was associated with better survival (Fig. 8c).

Table 7 Factors associated with distant metastasis-free survival in the study with StrTILs

		Univariate analysis			Multivariate analysis		
		Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value
ΔStrTIL		0.976	0.950-1.004	0.091	0.973	0.948-0.999	0.044
age	<50 years	1.000					
	≥50 years	1.545	0.690-3.455	0.290	0.892	0.344-2.318	0.815
grade	1-2	1.000					
	3	2.178	0.973-5.062	0.071	2.236	0.841-5.943	0.107
HR status	negative	1.000					
	positive	0.237	0.111-0.505	***<0.001***	0.169	0.072-0.398	***<0.001***
ypT	<2cm	1.000					
	≥2cm	2.107	0.964-4.602	0.062	3.854	1.520-9.775	***0.004***
ypN	negative	1.000					
	positive	1.644	1.562-17.147	***0.007***	6.984	2.011-24.261	***0.002***
pre-operative chemotherapy	anthracycline-based	1.000					
	platinum-based	0.737	0.297-1.830	0.511	0.741	0.269-2.044	0.563
	cyclophosphamide-based	0.642	0.284-1.451	0.287	0.961	0.388-2.379	0.931





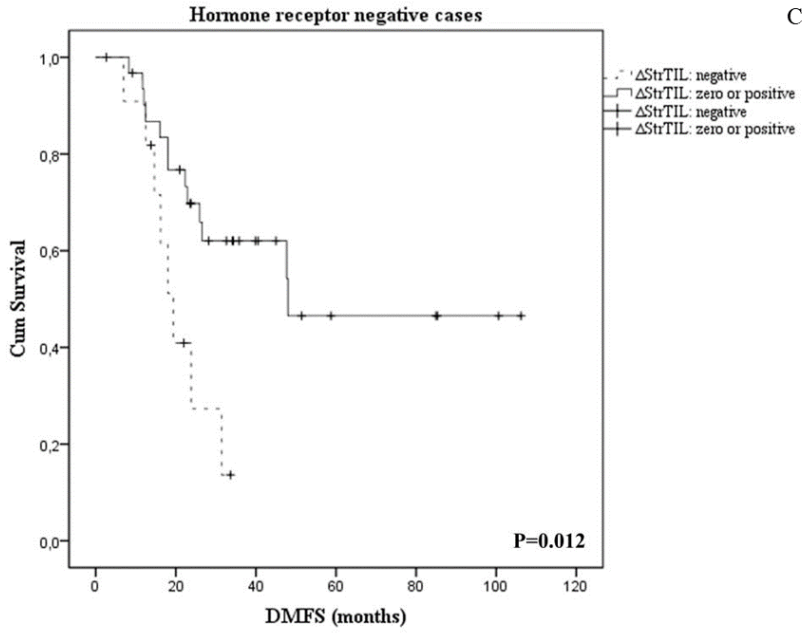


Figure 8 Kaplan-Meier curves of survival analyses
By analyzing the whole study cohort no significant correlation was detected between ΔStrTIL and DMFS; p=0.161 (**a**). The same result was observed in HR-positive cases too; p=0.339 (**b**).
In HR-negative cases the estimated median DMFS was significantly higher, if the ΔStrTIL was zero or positive (48.0 months; standard error: 8.7) compared to the cases where ΔStrTIL was negative (19.4 months; standard error: 2.4) (**c**).

Table 8. Prognostic value of pre-operative StrTIL, post-operative StrTIL and ΔStrTIL

	HR-negative cases			HR-positive cases		
	Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value
pre-StrTIL	1.022	0.997-1.049	0.088	1.028	0.872-1.212	0.745
post-StrTIL	0.974	0.948-1.000	***0.050***	1.009	0.980-1.040	0.545
ΔStrTIL	0.957	0.932-0.983	***0.001***	1.010	0.978-1.044	0.546

5. Discussion

Neoadjuvant endocrine therapy (NET) continued for one year resulted in pCR in 13.0% of the cases, two-thirds showed a significant regression and <10% presented a progression. *De novo* hormone resistance was revealed in five cases: one of them in which the tumor completely lost both ER and PgR expression and the other case in which the tumor has become HER2-positive after one-year of NET, as well as, three cases showed clinical progression during NET. One of the advantages of the neoadjuvant approach is that the benefits of the systemic therapy and thus the prognosis may be assessed *in vivo*. The potential therapy resistance could be revealed sooner than in adjuvant therapy.

The achievement of pCR is a predictor of excellent outcome after neoadjuvant chemotherapy, this also seems true for the subgroup of hormone-sensitive breast cancers after NET [5]. Although, the rate of pCR clearly increases as the NET period is extended, the rate of pCR is a rare event after NET [20, 21, 52, 53] compared to chemotherapy [5]. The first clinical trials with 3 months or shorter duration of NET published 0-3% pCR rates [21]. Hojo et al. found only a single case with pCR among 25 postmenopausal patients treated with exemestane for 6 months [20]. Allevi et al. [21] systematically treated three cohorts of 40 patients each with letrozole as NET for 4, 8 and 12 months, the rates of pCR in the 3 groups were 2.5, 5.0 and 17.5% ($p<0.04$), respectively. We experienced similar rate of pCR (13.0%) after one year of NET. Concerning the optimal duration of NET, when our article was published, the last 13th St. Gallen International Expert Consensus [54] recommended that NET should be continued until maximal response, while the next Consensus meeting in 2015 changed it to either 4-8 months or maximal response [55]. The optimum length of NET has not yet been exactly established, but currently all guidelines suggest surgery, when progression is experienced [2]. Though pCR after NET predicts excellent survival, the lack of complete response did not necessarily imply poor prognosis in this therapeutic approach. In our analysis with limited follow-up time, we experienced that the 82.6% (19/23) of patients in risk group 2 (stage IA-IIA residual cancer) were tumor progression-free. Furthermore, their estimated mean PFS was more than 8 years (97.5 months) similarly to the risk group 1. In contrast, the heavy post-treatment lymph node involvements (ypN2 or worse) was associated with poor prognosis.

To predict outcome, different approaches have been implemented. A change in Ki67 seems to be an early indicator of response, and may be utilized with this aim as soon as after 2 weeks of endocrine therapy [56]. Indeed, the study by Allevi et al. indicated that a decrease in Ki67 expression is manifested in the early phase of the treatment, and the expression does not

decline further on if the administration of letrozole is prolonged for 4, 8 or 12 months, despite the seemingly time-dependent tumor regression [21]. DeCensi et al. observed that Ki67 was a good predictor of the prognosis after short-term NET, with a 5.5-times higher risk of death in cases with a post-treatment Ki67≥20% [57]. The molecular background is that ET does not enhance the apoptosis of cancer cells, instead, hinders or decelerates cell proliferation. Ellis et al. on the basis of the long follow-up data of the P024 neoadjuvant endocrine trial developed a prognostic index ("preoperative endocrine prognostic index, PEPI") incorporating post-therapy tumor size, nodal status, grade, ER, Ki67 and the response to therapy [58]. The PEPI was validated in the independent IMPACT trial [59]. In good agreement with these findings, we found that the remaining cancer burden and post-treatment Ki67 were strong predictors of PFS.

The identification of endocrine sensitive tumors used to be difficult a couple of years earlier. Our results, in agreement with the data of Ellis et al. [58, 60] and Toi et al. [61] point to the fact that the most significant predictor of the response is the initial expression of ER. Although the high tumor grade and high Ki67 expression may be indicators of hormone resistance, these markers do not exclude a good effect of hormone therapy. Currently, a more sophisticated method for the prediction of hormone sensitivity is gene expression profiling being widely available for adjuvant treatment decision in node negative cases. Although there are promising results with multigene tests in 1 to 3 positive lymph node cases and neoadjuvant cases, but the evidence is still lacking for the recommendation of routine use [2, 8, 9, 62].

Few published data are available on NET in premenopausal patients. In the GEICAM/2006-03 study, the premenopausal patients were randomized to neoadjuvant chemotherapy *versus* exemestane therapy combined with goserelin [63]. While the treatment results were equivalent in the postmenopausal group, significantly more responses were observed in the chemotherapy arm than in the NET arm among the premenopausal patients (probably due to the lack of patient selection). Most of the previously reported NET studies were performed with aromatase inhibitors, but conflicting data recently appeared relating to their adjuvant use in premenopausal patients [64, 65]. According to our result, excellent therapy response can be achieved in premenopausal cases, however, the investigation of further biomarkers might be important in these cases, for instance *BRCA* mutation status. Nowadays, the role of young age, *per se*, as an indication for chemotherapy is less strongly endorsed given the growing appreciation for tumor biology as the determinant of outcome and the potential role for ovarian suppression [2, 9].

The investigation of *LAPTM4B* copy number by FISH in HR-negative cases showed an association of extra copy number and worse outcome in breast cancer treated with anthracycline-based neoadjuvant chemotherapy.

The patients who were resistant to neoadjuvant anthracycline-based therapy or responded to it but the tumor reduction was less than 50%, had significantly higher average *LAPTM4B* copy number/cell, than patients with higher tumor regression or pCR. In the adjuvant anthracycline-based treated cohort, the average gene copy number was higher where distant metastases were diagnosed during the follow-up period compared to the non-metastatic ones and patient with elevated *LAPTM4B* had worse DMFS.

The existence of the mammalian lysosomal-associated protein transmembrane family and its role in multidrug resistance have been known since the 1990s [66–68]. LAPTM4A was the first member and two others the LAPTM4B and LAPTM5 have been described until now [69, 70]. In the last few years, LAPTM4B received special attention and was widely investigated after that its overexpression was shown to be correlated with poor prognosis in several cancers [71, 72]. Kasper et al. [73] by analyzing different tumor types found that LAPTM4B was upregulated in 88% (23/26) of lung carcinomas, in 67% (18/27) of colon carcinomas, and in the majority of endometrial (30/44), breast (27/53) and ovarian (11/16) carcinomas.

The *LAPTM4B* gene is located in chromosome 8q22 and encodes two protein isoforms, LAPTM4B-35 and LAPTM4B-24, with molecular weights of 35 kDa and 24 kDa, respectively [74]. Mostly the 35-kDa isoform of LAPTM4B is overexpressed in numerous tumor types. In the last few years several putative oncogenic functions of LAPTM4B were identified. This transmembrane protein promotes cell survival, tumorigeneses, increases cell proliferation and drug resistance.

Li et al. found that the knockdown of *LAPTM4B* in MDA-MB-231 and BT549 cell lines leads to elevated nuclear localization of doxorubicin [30], thereby proving the lysosome membrane stabilizing properties of LAPTM4B, which is responsible for retaining and decrease nuclear localization of anthracycline by sequestering in cytoplasmic compartment. They observed also that elevated level of LAPTM4B and YWHAZ (another gene localized on 8q22) mRNAs was associated with shorter DMFS in women treated with adjuvant anthracycline chemotherapy, but in accordance with our findings, no association could be demonstrated between LAPTM4B alterations and treatment response in the non-anthracycline-treated cases. In another study, Li et al. demonstrated that LAPTM4B is required for lysosome homeostasis, acidification and function. By limiting lysosome-mediated cell death and promoting

autophagy the protein has a significant effect on cancer cell survival, including greater resistance to nutrient deprivation, hypoxia or doxorubicin-induced genotoxic stress [31].

A different study found that the interaction of the LAPTM4B-35 isoform with multidrug resistance protein-1 could result in increased drug efflux. In addition, LAPTM4B-35 promotes anti-apoptosis by the interaction of the PI3K/AKT signaling pathway [75]. Although more and more new LAPTM4B mechanisms of action have been described, several unanswered questions have still remained [76–78].

The prognostic value of higher average CEN8q or chromosome 8 polysomy in breast carcinomas is not clear. We have found significantly higher average CEN8q copy number in the group of patients with inferior response to anthracycline-based neoadjuvant therapy. Anna Batistatou et al. have found that polysomy-8 was present in 39% of HER2-positive and 30.2% of HER2-negative tumors. They also showed that the *MYC* gene amplification in the presence of chromosome 8 instability has distinct effects on patient outcome compared with *MYC* amplification with intact CEN8 [79]. We have analyzed the CEN8q and *LAPTM4B* copy numbers among HER2-positive and TNBC samples, but no difference was found. However, further analysis may be needed using higher number of cases.

By investigating the StrTIL before and after different mutagenic chemotherapy regimens, we observed similar TIL elevation in the three treatment groups. There are several possible explanations for this lack of difference. Although we used a classification of chemotherapy mutagenicity based on study performed in cell lines by Szikriszt et al. [43], currently there are no reliable measures of mutagenic capacity of these agents in human tumor samples. Hence we may not be sure that the difference seen in cell lines also holds for human tumors *in vivo*. It is also possible that the higher mutagenicity of a given agent, e.g. cisplatin, is compensated by another mechanism, such as the downregulation of the MHC [80]. While this is possible, it should be noted that platinum treatment was reported to induce the expression of the human leukocyte antigen in breast cancer [81]. Finally, it was suggested that in breast cancer, the main increase in therapeutic TIL response is driven by the gamma delta lymphocytes and not by the alpha beta lymphocytes, notably the former mechanism is typically not induced by neo-epitopes [82].

Several previous studies have evaluated the clinical importance of TILs in breast cancer.

Distribution of TIL is variable among breast cancer subtypes, higher TIL involvement was observed in TNBC and HER2-positive cases than in the ER-positive/HER2-negative subgroup as the incidence of lymphocyte predominant cases was also significantly less frequent [83]. The prognostic and predictive importance of baseline TILs at the diagnosis is

not a new concept in triple-negative or HER2-positive breast cancers. Denkert et al. investigated core biopsies from two neoadjuvant anthracycline/taxane-based studies with the result that the percentage of TIL was a significant independent parameter for pCR in both cohorts, independently from the subtype of the breast cancer; lymphocyte-predominant breast cancer responded, with pCR rates of 40% and 42% [84]. Loi et al. in node-positive, ER-negative/HER2-negative adjuvant anthracycline-treated breast cancer cases demonstrated that increasing lymphocytic infiltration was associated with excellent prognosis, and also that in HER2-positive breast cancer, an association exists between increasing lymphocytic infiltration and the magnitude of benefit of the chemotherapy regimens. Similarly to our results, they did not find significant prognostic association in the ER-positive/HER2-negative population, even if the luminal A and B subgroups as defined by Ki67 of 14% was investigated separately [83].

Lymphocyte predominant (or high-TIL) tumors is the term for tumors that contain more lymphocytes than carcinoma cells, the threshold is >50.0-60.0%; overall, the occurrence of TIL-rich cases is infrequent ~10% [51]. First Dieci et al. described that the chemotherapy-resulted high level of TIL in residual disease is a strong favorable prognostic factor in TNBC [85]. The correlation between post-treatment levels of TIL or ΔTIL and clinical outcome is less investigated. To our best knowledge, the only article about the prognostic role of the change in StrTIL levels between the pre- and post-treatment samples, as we did, is published recently by Pelekanou et al. [86]. In that study, 58 patients were involved who received neoadjuvant chemotherapy regardless of the HR status or used therapy regimen. They also found that increase in TIL count was associated with better relapse-free survival. Interestingly, 79.3% of their investigated cases were HR-positive. High post-treatment TIL levels were associated with better outcome in some studies [85, 87–89] whereas others did not find similar associations [90, 91]. Here we are confirming the observation that the increase of TIL ratio in the residual disease as a surrogate measure of anti-tumor immune activation may in fact reflect significant therapeutic benefit.

Currently, the most intensively developing and the most extensively investigated field in oncology is immuno-oncology. Immune checkpoint inhibitors are promising agents that are capable to block T-cell inactivation. There are many ongoing clinical trials with PD-1 inhibitors (pembrolizumab,) and PD-L1 inhibitors (durvalumab, atezolizumab, avelumab) in breast cancer. TIL status could serve as a predictive biomarker for immunotherapy with checkpoint inhibitors [92]. However, the clinical usefulness of chemotherapy-induced TIL in decision making about immunotherapy has been so far unclear.

6. Conclusions

6.1 One year NET results in a pCR rate of about 13% among HR-positive breast cancers. The response to NET is related to the expression of ER in the pre-therapy specimen while outcome after NET is related to the post-therapy tumor stage and Ki67 expression. The cases with stage IIB≤ residual tumor or Ki67 >15% have the worst PFS.

Long duration NET is effective and safe in cases of hormone sensitive breast cancer.

6.2 Analyzing *LAPTM4B* copy number may support future treatment decision and the use of alternative treatment modalities without anthracyclines should be considered for those patients whose cancer harbors extra copies of *LAPTM4B.*

6.3 By comparing the effects of different mutagenic pre-operative chemotherapy regimens on the percentage of stromal tumor infiltrating lymphocytes resulted in no significant differences. Further investigations are warranted to clarify the mutagenicity of various chemotherapy agents and their role in induction of antitumor immune response. Post-StrTIL status and the change of StrTIL after neoadjuvant chemotherapy may be used as new prognostic factors in HR-negative breast cancer.

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9. Appendix